Universidade do Minho
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Tese de Doutoramento em Ciências
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Professor Doutor Alberto Carlos Pires Dias

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DECLARATION

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Marta Raquel Martins Lima

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Day and night I went without sleep, trying to understand what goes on in this world. I saw everything God does, and I realized that no one can really understand what happens. We may be very wise, but no matter how much we try or how much we claim to know, we cannot understand it all.

Ecclesiastes 8:16-17

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To my grandfather - by passing away you revealed to me a precious secret to a Biologist: Life is everywhere and is never-ending... Thank you.

Contributions to the study of *Vitis vinifera* defence mechanisms against esca

ABSTRACT

Esca is a destructive disease of complex aetiology affecting grapevines worldwide and leading to great economic losses in viticulture. In the *Vinho Verde* Demarcated Region (North of Portugal), esca and young grapevine decline has been leading to important losses in wine production. The disease is widespread all over the region affecting all varieties of *Vinho Verde* grapevines, both old and young plants.

To study the putative response of *Vitis vinifera* to esca-related fungi a laboratory model was established, using cells suspension cultures of leaf explants of *V. vinifera* cv. Vinhão. These suspensions were elicited with an extract of the esca-related fungus *Phaeomoniella chlamydospora* and several defence mechanisms were studied. Elicitation of cell cultures with the fungal extract led to an increased phenolic production, including the *de novo* production of the viniferin type compounds ε- viniferin-2-glucoside, ε- viniferin-glucoside and a polymer of two ε-viniferin molecules. The antifungal activity of viniferins has been described in the literature. Also, the effect of the signalling molecules salicylic acid and methyl jasmonate in the defence response was analysed. Methyl jasmonate led to an increase of stilbenic and anthocyanin production, including the stimulation of the *de novo* production of the same viniferin type compounds as fungal elicitation. Early defence mechanisms, namely oxidative response, occurring in cells suspension cultures after fungal extract elicitation were also studied. The presence of a biphasic oxidative burst, typical of incompatible interactions, induced by fungal elicitation was shown. The influence of priming with the phytohormones salicylic acid and methyl jasmonate in the oxidative burst was also analysed. Although ROS accumulation seems to be potentiated in the first phase of thrburst by salicylic acid priming, priming with both salicylic acid and methyl jasmonate seems to be obstructive to ROS accumulation during the second phase of the oxidative burst. When studying the fungal induced biphasic oxidative burst using a calcium chelator and several calcium channel blockers, results indicated that oxidative response is

dependent on calcium availability and that the specific channel types from which calcium influx is originated are also important to induce oxidative burst. Cells suspension cultures were also used to study the putative roles of NADPH oxidase, catalase and peroxidase enzymes in the oxidative burst. Results indicate that NADPH oxidase is the primary ROS producer in the oxidative burst; also, early catalase activity does not seem to be altered by fungal elicitation, while peroxidase might play a role in the second phase of the burst of the oxidative response. Further results also indicate that calcium availability is necessary for NADPH oxidase activation. In addition, the ability of fungal elicitation to induce defence-related genes expression in cells suspension cultures was studied. Increased expression of genes encoding pathogenesis-related proteins (PR-6, PR-10, GLUC, CH3), and enzymes involved in the octadecanoid (LOX) and phenylpropanoid (PAL, STSY) pathways was shown. Overall, these results indicate that *V. vinifera* cv. Vinhão cells suspension cultures can be an important tool to study esca, since they allow to selectively study host defence response against esca-related fungi, without interference of external factors, leading to reliable results.

Field esca-affected material was also studied. The phenolic content of diseased, apparently healthy and healthy leaves of *V. vinifera* cv. Alvarinho was analysed. The total phenolic content was shown to be increased in diseased and apparently healthy leaves; furthermore, the phenolic content seemed to increase linearly from healthy to diseased leaves, with apparently healthy leaves showing an intermediate amount. Application of a PCA to HPLC data clearly separated diseased from healthy leaves (with apparently healthy leaves clustered in a medial position) and indicated the most important metabolites contributing to that difference. Of those, one metabolite was selected and identified as Kaempferol-3-glucoside. This compound was shown to be increased in diseased and apparently healthy leaves, increasing in a similar manner as that described above for total phenolics. In addition to phenolic content, the overall metabolic response towards esca was studied using NMR spectroscopy. Besides a huge increase in the phenolic compounds in diseased leaves, a decrease of carbohydrates was also detected, suggesting that diseased leaves are rerouting carbon and energy from primary to secondary metabolism. Methanol, alanine and GABA amounts were also shown to be increased in diseased leaves. Overall, these results indicate that *V. vinifera* cv. Alvarinho mounts a defence response towards esca, in spite of its recognized susceptibility to the disease.

Contribuições para o estudo de mecanismos de defesa de *Vitis vinifera* contra a esca

RESUMO

A esca é uma doença destrutiva de etiologia complexa que afecta videiras em todo o mundo e leva a graves prejuizos na viticultura. Na Região Demarcada dos Vinhos Verdes (Norte de Portugal), a esca e o declínio de videiras jovens tem levado a perdas importantes na produção vinícola. A doença encontra-se espalhada por toda a região, apresentando-se em todas as castas de Vinho Verde e afectando tanto videiras adultas como jovens.

Com o objectivo de estudar a existência de uma resposta de *Vitis vinifera* contra fungos associados à esca, criou-se um modelo laborarial a partir de explantes foliares de *V. vinifera* cv. Vinhão, estabelecendo-se culturas de células em suspensão. Estas suspensões foram eliciadas com um extracto do fungo *Phaeomoniella chlamydospora* (associado à esca) e vários mecanismos de defesa foram estudados. A eliciação das culturas de células com um extracto do fungo levou ao aumento da produção fenólica, incluindo a produção *de novo* dos compostos tipo viniferina: ε-viniferina-2-glucósido, ε-viniferina-glucósido e um polímero de duas moléculas de ε-viniferina. A actividade antifúngica das viniferinas já foi descrita na literatura. Foi analisado o efeito das moléculas sinalizadoras ácido salicílico e metiljasmonato nesta resposta de defesa. O metiljasmonato induziu o aumento da produção de compostos stilbénicos e antocianinas; inclusivamente estimulou a produção *de novo* dos mesmos compostos tipo viniferina induzidos pela eliciação fúngica. Também foram estudados mecanismos de defesa precoces, nomeadamente resposta oxidativa, que ocorrem nas suspensões celulares após eliciação com o extracto fúngico. Foi demonstrada a presença de um *burst* oxidativo bifásico, típico de interacções incompatíveis, induzido pela eliciação com fungo. A influência da indução com as fitohormonas ácido salicílico e metiljasmonato no *burst* oxidativo foi analisada. Apesar da acumulação de espécies reactivas de oxigénio parecer ser potenciada na primeira fase do *burst* pela indução com ácido salicílico, tanto a indução com este como com metiljasmonato parece obstruir a acumulação de espécies reactivas de oxigénio durante a segunda fase do *burst* oxidativo. O uso um quelador de cálcio e vários bloqueadores de canais de cálcio na análise da resposta oxidativa induzida pela eliciação fúngica, indicou que o *burst* oxidativo depende da

disponibilidade de cálcio e que os tipos de canais através dos quais é originado o influxo de cálcio também são importantes para induzir a resposta oxidativa. Também se estudaram nas culturas de células em suspensão as possiveis funções das enzimas NADPH oxidase, catalase e peroxidase no *burst* oxidativo. Os resultados indicaram que a NADPH oxidase é a fonte primária de espécies reactivas de oxigénio na resposta oxidativa; por outro lado, a actividade da catalase não pareceu ser alterada pela eliciação fúngica enquanto a peroxidase parece poder desempenhar um papel na segunda fase do *burst* oxidativo. Os resultados também sugerem que a disponibilidade de cálcio é necessária para a activação da NADPH oxidase. Adicionalmente, foi estudada a capacidade da eliciação fúngica induzir a expressão de genes associados à defesa nas culturas de células em suspensão. Os resultados revelaram uma expressão aumentada dos genes que codificam proteínas relacionadas com a patogénese (PR-6, PR-10, GLUC, CH3) e enzimas envolvidas nas vias octadecanoica (LOX) e fenilpropanoide (PAL, STSY). No geral, estes resultados indicam que as culturas de células de *V. vinifera* cv. Vinhão podem ser uma ferramenta importante para estudar a esca, uma vez que permitem analisar selectivamente a resposta de defesa do hospedeiro contra fungos associados à esca, sem interferência de factores externos e levando a resultados fidedignos.

Também foi estudado material de campo afectado pela esca. O conteúdo fenólico de folhas doentes, aparentemente sãs e sãs de *V. vinifera* cv. Alvarinho foi analisado. Foi demonstrado que o conteúdo fenólico total se encontra aumentado nas folhas doentes e aparentemente sãs, e que o conteúdo fenólico parece aumentar linearmente das folhas sãs para as doentes, com as folhas aparentemente sãs mostrando uma quantidade intermédia. A aplicação de uma PCA aos dados de HPLC separou claramente as folhas sãs das doentes (com as folhas aparentemente sãs agrupadas numa posição medial) e indicou os metabolitos mais importantes que contribuem para essa diferença. Desses metabolitos, um foi seleccionado e identificado como kaempferol-3-glucósido. Este composto mostrou-se aumentado nas folhas doentes e aparentemente sãs, aumentando de um modo similar ao descrito para os fenólicos totais. Além do conteúdo fenólico, a resposta metabólica geral contra a esca foi estudada usando espectroscopia RMN. Além de um grande aumento dos compostos fenólicos nas folhas doentes também foi detectada uma diminuição dos carbohidratos, sugerindo que as folhas doentes reencaminham o carbono e energia do metabolismo primário para o secundário. Nas folhas doentes, também foi detectado um aumento das quantidades de metanol, alanina e GABA. No geral, estes resultados indicam que *V. vinifera* cv. Alvarinho monta uma resposta de defesa contra a esca, apesar da sua reconhecida susceptibilidade à doença.

CONTENTS

ABBREVIATURES

1D	One dimension
2D	Two dimension
4-MN	4-methoxy-α-naphtol
AC	Adenosine monophosphate cyclise
ACT	Actin
ahl	Apparently healthy leaves
ANOVA	Analysis of variance
atm	Atmosphere
Avr	Avirulence gene
bp	Base pairs
C	Carbon
ºC	Celsius degrees
Ca^{2+}	Calcium
$[Ca^{2+}]_{cyt}$	Cytosolic calcium concentration
cADP	Cyclic adenosine diphosphate
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CDPKs	Ca^{2+}-dependent protein kinases
CH3	Class III chitinase
Cl^-	Chlorine
Cm^2	Square centimetre
CoA	Coenzyme A
cv.	Cultivar
CVRVV	Comissão de viticultura da região dos vinhos verdes

DAD	Diode array detection
DAG	Diacylglycerol
DCF	Dichlorofluorescein
dl	Diseased leaves
DPI	Diphenyleneiodonium chloride
dwb	Dry weight biomass
ε	Extinction coefficient
EGTA	Ethylene glycol-bis(2aminoethylether)-N,N,N',N'-tetraacetic acid
ESI	Electrospray ionization
FIDs	Free induction decays
Fmed	*Fomitiporia mediterranea*
Fop	*Fomitiporia punctata*
FT	Fourier transformation
g	Gram
g	Gravity acceleration
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	Glutamate decarboxylase
GC	Guanosine monophosphate cyclise
GLUC	β-1,3-glucanase
G-protein	Guanosine triphosphate-binding protein
H^+/ H^1	Hydrogen (proton)
H_2DCF	Hydrodichlorofluorescein
H_2DCF-DA	Hydrodichlorofluorescein diacetate
H_2O_2	Hydrogen peroxide
hl	Healthy leaves
HPLC	High performance liquid chromatography
HR	Hypersensitive response
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IP_3	Inositol-1,4,5-triphosphate
ISR	Induced systemic resistance
JA	Jasmonic acid
K^+	Potassium
kV	Kilovolts
l	Litre

LC	Liquid chromatography
LOX	Lipoxygenase
lysoPC	Lysophosphatidylcholine
M	Molar
m^2	Square metre
MAPKs	Mitogen-activated protein kinases
MeJ	Methyl jasmonate
mg	Milligram
MHz	Megahertz
ml	Millilitre
µl	Microlitre
mM	Millimolar
mm	Millimetre
µm	Micrometre
µmol	Micromole
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
ms	Millisecond
µs	Microsecond
N	Nifedipine
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
ng	Nanogram
nkatal	Nanokatal
nm	Nanometre
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
O_2	Oxygen
O_2^-	Superoxide anion
PAL	Phenylalanine ammonia lyase
Pal	*Phaeoacremonium aleophilum*
PAMPs	Pathogen-associated molecular patterns

PC	Principal component
PCA	Principal components analysis
Pch	*Phaeomoniella chlamydospora*
PDA	Potato dextrose agar
PLA	Phospholipase A
PLC	Phospholipase C
pM	Picomolar
PMEs	Pectin methylesterases
ppm	Parts per million
PR	Pathogenesis related
PR-10	Class 10 PR protein
PR-6	Class 6 PR protein
PVP	Polyvinylpirrolidone
R	Resistance protein
R	Resistance gene
RDVV	Região demarcada dos vinhos verdes
ROS	Reactive oxygen species
rpm	Rotation per minute
RR	Ruthenium red
s	Second
SA	Salicylic acid
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
sp.	Specie
spp.	Species
SSA	Succinic semialdehyde
STSY	Stilbene synthase
SW	Spectral width
TFs	Transcription factors
t_m	Mixing time
TMV	Tobacco mosaic virus
TOCSY	Total correlation spectroscopy
TPPI	Time proportional phase incrementation
TSP	Trimethyl silane propionic acid
u	Units
Un	Unknown

UV	Ultraviolet light
V	Volts
VOCs	Volatile organic compounds
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1 - GENERAL INTRODUCTION

In the previous page: illumination by Jean Bourdichon, from *Horae ad usum romanum – Grandes heurs d'Anne de Bretagne* (1503-1508), Tours – France; Bibliothèque nationale de France, Département des Manuscrits, Division occidentale (cote Latin 9474, folio 156). Accessed from Mandragore, base des manuscrits enluminés de la Bibliothèque nationale de France (http://mandragore.bnf.fr/html/accueil.html) on 12th January 2009.

1.1 – Viticulture and the Demarcated Region of Vinhos Verdes – a brief historical overview

The grapevine is not only a major horticulture crop nowadays, but also is intimately linked to the development of mankind societies since ancient times (This *et al.*, 2006). Its main product, the wine, was considered divine by several human cultures: it was attributed to Osiris by Egyptians, to Noah by Armenians, to Dionysus by Greeks and to Bacchus by Romans (Osório, 2002; This *et al.*, 2006).

Although it is known that the wild form of grapevine – *Vitis silvestris* – exists for more than 1 million years, it is not clear when domestication of grapevine and wine making started. Nonetheless, it is thought that started with the beginning of sedentary lifestyle, about 7000 b.C. (Osório, 2002). Archaeological and historical evidence suggest that first-domestications started in the Near-East; seeds of domesticated grapes (*V. vinifera*) found in Georgia and Turkey dated from the Neolithic. It is thought that viticulture dispersed from these first-domestication sites to adjacent regions. By 3000 b.C. viticulture had reached Mesopotamia and Egypt, and spread further around the Mediterranean through the action of main civilizations: Assyrians, Phoenicians, Greeks, Romans, Etruscans and Carthaginians (Surico, 2000; This *et al.*, 2006). Romans spread *V. vinifera* culture from the Mediterranean to North, reaching inland regions such as Germany; grapevine was cultivated throughout Europe by the end of the Roman Empire (This *et al.*, 2006). In the Middle Ages and Renaissance, it was mostly the Christian religious orders that maintained and spread the viticulture to the farthest regions of Europe; also, the spreading of Islam to North Africa, Spain and

Middle-East led to the dispersal of grapevine culture (Surico, 2000; This *et al.*, 2006). In the XVI century, missionaries introduced *V. vinifera* in the New World; in the XIX century grapevine was introduced to South Africa, Australia and New Zealand (Surico, 2000; This *et al.*, 2006). Today, viticulture is spread throughout the 5 continents: it is responsible for the subsistence of 50 million individuals and occupies about 8 million hectares worldwide (Osório, 2002).

It is known that since the III century b.C. viticulture is practiced in the Northwest region of Portugal. This region, called *Entre-Douro-e-Minho*, has an altitude below 700 meters and is crossed by several river valleys (of the rivers: Minho, Lima, Cávado, Ave, Sousa, Tâmega and Douro); filled with natural compartments and plentiful water has always allowed the culture of several agricultural products (Osório, 2002). The Demarcated Region of *Vinhos Verdes* (RDVV – *Região Demarcada dos Vinhos Verdes*), almost coincident with the *Entre-Douro-e-Minho* region, is limited in the North by Minho river, in the South and West by mountainous regions, and in the East by the Atlantic Ocean; it is the greatest viticulture region of Portugal, and one of the greatest of Europe, occupying almost 7 thousand square kilometres and about 35 thousand hectares of cultivated vine (Osório, 2002).

However, until the XII-XIII century the viticulture in this region remained underdeveloped, mainly for familiar consumption only. After the XII century, the demographic and economic developments and the increase in the commerce of agricultural products led to the expansion of viticulture and the wine became an important font of income (CVRVV, copyright 1997-2003a). During the Middle Ages, not only the religious orders were the main keepers of viticulture in this region, but also the Crown (between the centuries XII-XIV) gave an important contribution to viticulture development (Osório, 2002). In the XIV century the wines produced in this region (*Vinhos Verdes*) were already exported to England, Flanders, Germany and North Sea (CVRVV, copyright 1997-2003a; Osório, 2002). In the XVII century, the intensification of wine exportation to Brazil and Portuguese islands and colonies overseas led to an extension of viticulture in the *Vinho Verde* region; nonetheless, during the Discoveries period, the Portuguese wine

commerce and viticulture decreased in consequence of increased attention and commercialization of products originated from Brazil and India (Osório, 2002).

In the mid XVIII century, the Portuguese viticulture was again stimulated by the creation of *Companhia Geral da Agricultura das Vinhas do Alto Douro*, a company that regulated wine export of wines produced in the North of Portugal. However, a few years later, was created the *Sociedade Pública de Agricultura e Comércio da Província do Minho*, a society that regulated the wine market of the Northwest region with the objective of increasing the *Vinho Verde* commerce (CVRVV, copyright 1997-2003b; Osório, 2002). In the XIX century, the epidemics of powdery mildew and phylloxera affected all European vines originating great losses; because the Northwest region of Portugal was less affected, the exports of *Vinho Verde* increased, mainly to France (Osório, 2002).

In the beginning of the XX century, after the epidemics of phylloxera, powdery and downy mildew, it became apparent throughout Europe the necessity to control the authenticity and quality of wine. With this objective, Portugal published in 1908 a law recognizing officially the demarcation of different Portuguese wine regions, including the RDVV divided in 5 sub-regions (Monção, Lima, Amarante, Basto and Braga), due to the existence of differences in cultural practices, grapevine varieties, and types of wine (CVRVV, copyright 1997-2003b; Osório, 2002). Later, in 1926, was published the regulation of production and commerce of *Vinhos Verdes* (*Regulamento de Produção e Comércio dos Vinhos Verdes*) that confirms the delimitation of RDVV and includes the Penafiel sub-region. This regulation, defines the geographical limits of the wine region, characterizes its wines, defines rules for the production, certification and commerce of *Vinhos Verdes*, and creates the Viticulture Commission of the *Vinhos Verdes* Region (CVRVV – *Comissão de Viticultura da Região dos Vinhos Verdes*) (Osório, 2002). The analytical characteristics of the wine for export and internal consumption were defined in 1935 and 1937, respectively (Osório, 2002).

The denomination of origin "*Vinho Verde"* was internationally recognized after the acceptance of the report claiming this denomination of origin, in 1949, by the *Office International de la Vigne et du Vin* in Paris, and after the international registry of this denomination of origin was recognized, in 1973, by World Intellectual Property Organization in Geneva (CVRVV, copyright 1997-2003a; Osório, 2002). In the same manner, it was important the creation, in 1959, of the *Selo de Origem* (seal of origin), as a mean to guaranty the origin and quality of *Vinho Verde*. Also of considerable significance for the RDVV was the diversification of the wine-derived products of this region when denomination of origin was recognized for brandies of wine or bagasse origin, in 1984 (CVRVV, copyright 1997-2003b; Osório, 2002).

In 1985, Portugal enters the European Economic Community (today's European Union) and several laws concerning viticulture were reformulated to approach the community regulations; for instances: it was published the list of recommended and authorized varieties, and for each were established the minimum and maximum percentages, respectively. It should be noticed that both Alvarinho and Vinhão are recommended varieties of RDVV. In 1992, the statutes of the RDVV were published, finally defining all aspects concerning the RDVV: geographical delimitation, soil nature, grapevine varieties (recommended and authorized and their allowed percentages), cultural and vinification practices, and chemical and organoleptic characteristics of the products, among others aspects (CVRVV, copyright 1997-2003b; Osório, 2002). In this period, in 1986, it was created an experimental unit in a farm localized in Arcos de Valdevez, the EVAG (*Estação Vitivinicola Amândio Galhano*), with the objective of improving the viticulture of RDVV, by studying several aspects of cultural practices (CVRVV, copyright 1997-2003b). Later, in 2001, the RDVV was reorganized in 9 sub-regions (Figure 1.1): Amarante, Ave, Baião, Basto, Cávado, Lima, Monção, Paiva and Sousa (CVRVV, copyright 1997-2003b; Osório, 2002).

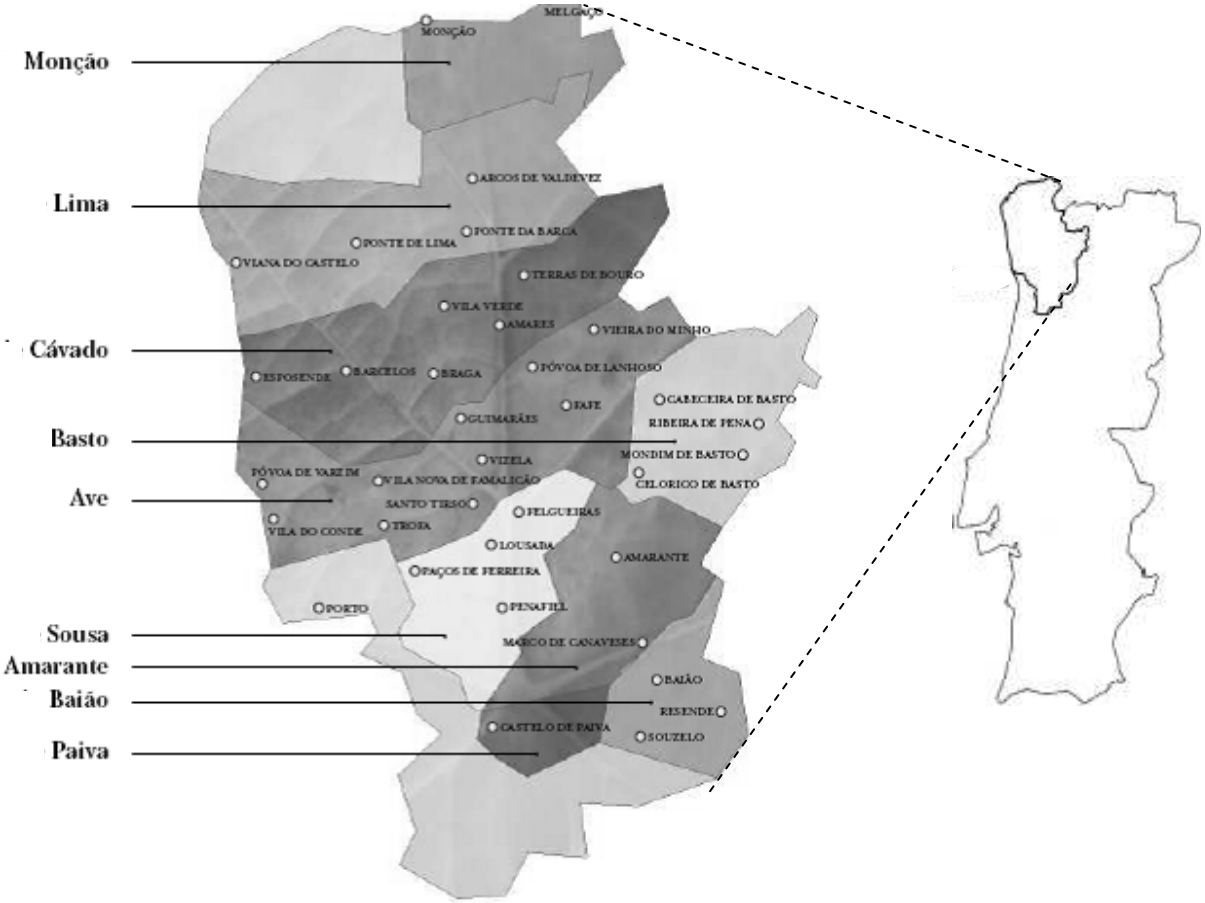


Figure 1.1 – RDVV in the Northwest of Portugal (adapted from Osório, 2002).

1.2 – Esca disease

For almost all grapevine diseases a treatment was found, including for the epidemics of phylloxera, powdery and downy mildew. Today, esca disease presents a major problem worldwide, for which no appropriate treatment has been found (Surico, 2000). But esca is not a new disease: it has been present for centuries in viticulture. Esca-like symptoms were known in ancient times, since they are referenced in both ancient Greek and Latin works; also, medieval works give a good description of these symptoms (Graniti, 2006; Mugnai *et al.*, 1999). However, in the last years, the disease has been presenting itself in a *more destructive manner* and, many times, is preceded or accompanied by other debilitating syndromes (Surico, 2000).

The word *esca* is also very old. In Latin means food or aliment, but it can also mean bait, lure or allurement; in some modern languages it came to signify tinder (Graniti, 2006; Surico, 2000). Indirectly, esca refers to the carpophores of wood-rotting fungi, such as *Fomes fomentarius* and *Fomes (Phellinus) igniarius*. These fungi were used to start fires: they grew in and decayed wood of several woody plants, including grapevine, giving rise to an easily flint-ignited material; the rotted wood burned slowly and kept flameless burning fires (Graniti, 2006; Mugnai *et al.*, 1999). In Greek, *yska* meant the rotted wood or the fungus itself (Surico, 2000). Finally, the name esca was proposed for the disease (previously known by many different names, e.g., *feuilletage* or apoplexy) in 1926 by Viala, since the disease had been associated with *P. igniarius*, which in some countries was called esca (among other tinder-related fungi) (Graniti, 2006).

Today, esca affects grapevines worldwide and the incidence of the disease has been increasing dramatically. In a survey study in Austria, the presence of grapevines showing esca symptoms was about 1.3% at the beginning of the 6 years study, and increased in average 2.7% per year, although an increase up to 20% was registered in some vineyards (Reisenzein *et al.*, 2000). A study in Spain also revealed dramatic increases in esca incidence (Redondo *et al.*, 2001). Some regions are severely affected by esca, for example Italy with an estimated incidence reaching 90-100% in some regions (Marchi, 2001; Mugnai *et al.*, 1999; Surico *et al.*, 2004; Surico *et al.*, 2000). But the disease is present in many other countries such as: Australia (Edwards *et al.*, 2001; Pascoe and Cottral, 2000), California (USA) (Eskalen and Gubler, 2001), Turkey (Ari, 2000; Köklü, 2000), Greece (Rumbos and Rumbou, 2001), and France (Peros *et al.*, 2000). In Portugal esca disease was also reported and its estimated incidence is of 20% (Mugnai *et al.*, 1999). Grapevine decline symptoms were registered in central and southern regions (Rego *et al.*, 2000), as well as in the northwest, in the RDVV (Chicau *et al.*, 2000).

It has been suggested that the increase in esca incidence, since the 80's of the last century, is related to new cultural practices. Extensive establishment of new vineyards (many times with infected material), changes in nursery and cultural practices leading to low quality nursery material, reduced sanitary care of rootstocks and vine propagation material, poor protection of pruning wounds, untreated frost lesions and use of less efficient fungicides since sodium arsenite was banned, seem strongly related to the dissemination of esca disease, formerly limited to old vines and controlled by simple sanitary practices and arsenite treatments. Also, climatic changes, particularly variation in precipitation, may have contributed to the boost in esca incidence (Graniti *et al.*, 2000; Surico *et al.*, 2006).

Up until now, no effective control strategy for esca could be found. Several control methods have been assayed since the banishment of sodium arsenite treatments: fosetyl-Al foliar treatments, application of triazolic fungicides, spraying with fungicides at pruning, painting with triazoles, application of fungicides by syringe injection, protection of wounds with

Trichoderma viridae, and hot water treatment of nursery materials. However, no satisfactory treatment was found; fosetyl-Al seems promising, but most of the treatments tested showed no effect in vine protection against esca (Di Marco *et al.*, 2000).

Hence, today, the control of esca still relies in traditional cultural methods to reduce losses and inhibit spreading of the disease. These practices consist mainly in protecting wounds of whatever origin (frost, pruning, biotic attack) and treat them with fungicides, marking infected plants and use separate pruning materials for healthy and infected vines, remove infected pruning residues from the vineyard and uproot and dispose of all dead infected plants (Di Marco *et al.*, 2000; Mugnai *et al.*, 1999). The development of an effective method for esca control depends on the progress made in understanding the disease: the complex interaction between esca-related fungi, grapevine and environmental factors (Di Marco *et al.*, 2000).

1.2.1 – Symptoms

Esca disease provokes both internal and external symptoms in affected grapevines. The disease can develop in a chronic or acute form, and the complexity of the disease becomes even more evident with the unpredictable (and still unexplained) discontinuity of external symptoms from year to year (Surico *et al.*, 2000).

Chronic esca symptoms can appear in the trunk, branches, leaves and berries. In the trunk and branches the most common symptom is white rot, usually associated to a pruning wound, and that extends into the woody tissue and can spread both up and down inside the trunk, and/or reach the surface causing cracks along the trunk. The rotting process softens the tissue turning the wood into a light-coloured spongy mass; in cross section it presents a dark line (black or brown) separating rotted from non-rotted tissue, as can be seen in Figure 1.2 (Mugnai *et al.*, 1999).

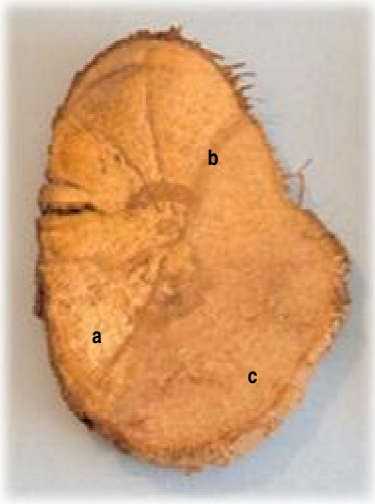


Figure 1.2 – Cross section of a *V. vinifera* cv. Alvarinho trunk showing white rot (a) delimitated with a dark line (b) from healthy woody tissue (c) (adapted from Felgueiras, 2006).

The white rot can be accompanied by other deteriorating symptoms (Figure 1.3): black or dark-brown streaks (appearing as small spots in cross section and scattered though the wood or in small clusters), pink-brown or dark red-brown areas on the margin of decayed/necrotic tissue (often developing from black spots), and brown areas of varying textures (often associated with large wounds) that intermingle with the above mentioned types of discoloration (Mugnai *et al.*, 1999).

Figure 1.3 - Cross section of a *V. vinifera* cv. Alvarinho trunk showing black spots (a) and pink-brown areas (b) (adapted from Felgueiras, 2006).

In the leaves (Figure 1.4), small chlorotic spots expand between the veins or along leaf margins and coalesce to fill the entire leaf, except a small line of healthy tissue along the main veins. This chlorotic tissue often necrotize turning to yellow-brown or red-brown colour; the necrotic tissue may dry and detach, with leaf margins becoming irregular. Clearing, puckering, glistening and distortion of leaf are symptoms less common (Mugnai *et al.*, 1999).

Figure 1.4 – Esca foliar symptoms in *V. vinifera* cv. Alvarinho (from Felgueiras, 2006).

In berries (Figure 1.5), symptoms consist of small spots, of brown, violet or purple colour, that appear scattered on the berry skin and are irregularly distributed or in longitudinal bands; in California, berries symptoms are called “black measles”. Severe spotting can be accompanied by skin cracks and, rarely, the spots can cover the entire berry. The spots are thought to be groups of necrotic cells near the end portion of a xylem vessel, affecting only the epidermal and hypodermal layers, and not the underlying tissues. Inside the grape cluster, the severity of symptoms may vary from berry to berry. Severely symptomatic berries dry up or are colonised by fungi or bacteria

and rot. Diseased vines may show late ripening of grape clusters, with grapes containing low sugar content and altered flavour; also, the berries may fail to ripen in consequence of wilt of distal parts (Mugnai *et al.*, 1999).

Figure 1.5 – Esca berry symptoms in *V. vinifera* cv. Alvarinho; enhancement of some berries shown in bottom left corner of the figure (adapted from Felgueiras, 2006).

The acute esca, also called apoplexy, is characterized by sudden and rapid wilt of entire vines or, rarely, of single branches (Figure 1.6). Healthy leaves can dry up in a few days. Usually, this violent form of the disease manifests in midsummer, particularly in dry, hot weather following rainfall (Mugnai *et al.*, 1999).

Figure 1.6 – Esca affected *V. vinifera* cv. Alvarinho branch completely dry (from Felgueiras, 2006).

1.2.2 – Aetiology

The aetiology of esca has been researched for more than a century, since 1898. Three periods of intensive research can be distinguished: the first took place from 1898 until 1926, the second started in 1957 and continued for two years, until 1959; and finally a third period that started in 1987 and is active until today (Mugnai *et al.*, 1999).

In the first period, works of Ravaz, Vinet and Viala suggested that fungal infection was on the basis of esca. The fungi *P. igniarius* and *Stereum hirsutum* were isolated from esca affected vines, and species of *Cephalosporium* and *Acremonium* were able to produce esca internal symptoms (Chiarappa, 2000).

In the second period, Hewitt drew attention to discontinuity of symptoms from year to year, and Chiarappa consistently isolated *P. igniarius* and

Cephalosporium sp. from decayed vine wood and reproduced wood decay symptoms using these fungi (Chiarappa, 2000).

In the third period of research Larignon and Dubos suggested that a succession of fungi after colonization by *Cephalosporium* sp. and *Eutypa lata*, was the cause of esca. During this period other fungi associated with wood decay were isolated and identified, and the spreading mode of these fungi were studied (Chiarappa, 2000). In spite of all the years dedicated to the study of esca, the aetiology of the disease remains controversial and partially unexplained. It was proposed in 1999 in the first International Workshop on Grapevine Trunk Diseases – Esca and Grapevine Decline, in Sienna – Italy, that *esca was a complex disease and the syndrome of esca as a whole was caused by a number of factors interacting with each other and involving more than one causal micro-organism* (Surico *et al.*, 2006).

1.2.2.1 – Main fungi associated and their pathogenicity

Several fungi have been associated with esca disease. Among the most frequently associated fungi in esca affected vines are: *Fomitiporia mediterranea* (*Fmed*) or *Fomitiporia punctata* (*Fop*; previously misidentified as *P. igniarius* according to Mugnai *et. al.* (1999)), *Phaeomoniella chlamydospora* (*Pch*; former *Phaeoacremonium chlamydosporum*, former *Cephalosporum* sp.), and *Phaeoacremonium aleophilum* (*Pal*; previously *Acremonium* sp.). Other *Phaeoacremonium* sp. such as *P. angustius* and *P. inflatipes* are also consistently isolated from esca infected vines, although with a lower frequency than the previously mentioned fungi (Graniti *et al.*, 2000; Mugnai *et al.*, 1999; Surico *et al.*, 2006).

In RDVV, *Pch*, *P. angustius* and *Phaeoacremonium* spp. are the fungi most frequently isolated from esca diseased vines (Chicau *et al.*, 2000; Felgueiras, 2006).

It has been suggested that basidiomycetes such as *Fop* causes the white rot, while the deuteromycetes *Pch* and *Pal* are responsible for the brown wood-streaking, discoloration of woody portions, foliar symptoms and decline of young grapevines (Graniti *et al.*, 2000; Mugnai *et al.*, 1999).

In an attempt to confirm the role of these fungi in esca, numerous *in vitro* and *in planta* studies have been conducted in several laboratories around the world.

It was shown that *Pch* produces pectic enzymes which may help the fungus to colonise the plant tissue (Marchi *et al.*, 2001). Also, *Pch*, *Pal* and *Fmed* have been show to produce tannase, laccase and peroxidase enzymes, which may enable them to macerate cell walls and woody tissue or to tolerate plant's defensive compounds by oxidizing phenolics (Bruno and Sparapano, 2006).

Also, it was shown that *Fop*, *Pch* and *Pal* are capable of producing several phytotoxins (Tabacchi *et al.*, 2000). Particularly scytalone and isosclerone, as well as pullulans, produced by *Pal* and *Pch* were shown to produce esca-like symptoms in healthy grapevine detached leaves and berries (Bruno and Sparapano, 2006a; Bruno *et al.*, 2007; Evidente *et al.*, 2000; Sparapano *et al.*, 2000c).

Co-culturing grapevine *calli* with *Pch*, *Pal* and *Fop*/*Fmed* led to reduced *calli* growth, as well as induced browning and necrosis (Bruno and Sparapano, 2006a; Sparapano *et al.*, 2000c; Sparapano *et al.*, 2001b). In addition, co-culturing these fungi with grapevine *in vitro* plantlets induced esca-like symptoms in leaves (Sparapano *et al.*, 2001b). The inoculation of detached healthy grape berries with *Pch* and *Pal* also led to the appearance of typical esca-like lesions in 4 to 5 days (Gubler *et al.*, 2004). Furthermore, the capacity of *Pch* and *Pal* to produce wood decay symptoms was observed in laboratory, by inoculating these fungi in wood blocks obtained from healthy grapevine plants (Larignon and Dubos, 1997).

The majority of esca symptoms, both internal (in the trunk) and external (in leaves and berries) were successfully reproduced within 3 years after inoculation of esca-free grapevines with *Pch*, *Pal* and *Fop*. Besides, because the fungi were inoculated singly and in all possible combinations, it was indicated that several symptoms are commonly produced by all 3 fungi, such as wood streaking and foliar chlorosis, while other symptoms are characteristically induced by each type of fungi, such as black goo and black

measles by deuteromycetes – *Pch*/*Pal* – and white rot by basidiomycetes – *Fop* - (Sparapano *et al.*, 2000b; Sparapano *et al.*, 2001a). The capacity of *Fop* as a wood-rot inducer alone (without the presence of *Phaeoacremonium* spp.) had already been observed in the field, by inoculating *Fop* in both adult and young healthy grapevines through wounds. Wood decay symptoms including white-rot developed within 2 years after inoculation, but the first signs of wood-rot were observed as soon as 6 months post inoculation (Sparapano *et al.*, 2000a).

Isosclerone and scytalone were also shown to be produced *in planta*, since these compounds were isolated from xylem sap, leaves, rachides and berries of grapevines naturally infected with *Pch*, *Pal* and *Fmed*, and that showed both internal and external esca symptoms. Pullulans was also recovered from xylem sap. Furthermore, the absorption of collected xylem sap by healthy detached leaves induced the appearance of esca-like symptoms (Bruno and Sparapano, 2006b; Bruno *et al.*, 2007).

Although successful results were reached in the previously mentioned works, not all attempts to prove the pathogenicity of the esca-associated fungi were fortunate, particularly concerning the reproduction of esca external symptoms *in vivo*. Moreover, esca-related deuteromycetes have been isolated from asymptomatic plants raising the question if these fungi *are endophytes, latent pathogens, weak pathogens or true vascular pathogens* (Graniti *et al.*, 2000; Surico *et al.*, 2006) This may indicate that other factors are needed for developing esca syndrome besides the presence of at least the above mentioned esca-associated microorganisms. These factors are still unclear, but may include vine age, variety susceptibility, site and time of infection, plant's defence response and environmental factors such as water and nutrient availability, temperature and humidity, among others (Graniti *et al.*, 2000). As follows, several hypotheses have been proposed to explain the appearance of symptoms (and their discontinuity from year to year).

The different wood decay symptoms are the result not only of fungi excreted lytic enzymes and/or toxins (leading to degradation and/or necrosis of woody

tissues) but also of physiochemical changes induced by the entrance of air and water through the wounds, the host's reactions to the wounds and invading pathogens, host's growth-inducing substances that induce tylosis or host's secreted gels and gums that lead to vascular occlusion, as well as host's phytoalexins that may cause tissue's necrosis (Mugnai *et al.*, 1999).

The causes of esca external symptoms seem to be mainly related to the translocation of fungal toxins or host's phytoalexins from decayed wood to leaves and berries, through sap flow (Bruno *et al.*, 2007; Mugnai *et al.*, 1999). It was also suggested that foliar symptoms, particularly wilting, could result from non-functional xylem (Surico *et al.*, 2006). In addition, the appearance of external symptoms seem to be related to weather conditions, since rainy seasons appear to favour the manifestation of foliar symptoms, contrarily to dry seasons (Marchi *et al.*, 2006).

The expression of the acute form of esca seems to be related to weather conditions, usually manifesting when dry, hot weather follows a rainfall. Also, vine age appears to be an important factor in apoplexy, since the fulminating form of the disease is mainly restricted to older vines, although there are exceptions (Mugnai *et al.*, 1999; Surico *et al.*, 2006).

1.2.2.2 – Model of esca development

A model of the development of esca was proposed in 2001, integrating almost all the information obtained about esca until that moment (Surico, 2001). The proposed scheme is shown in Figure 1.7, and includes the complexity of the disease that involves different syndromes: young grapevine decline, young esca, white-rot and esca proper.

Young grapevine decline (also called Petri grapevine decline) is characterized by brown wood streaking, wood gummosis or xylem darkening, and affected vines may experience decline, wilting, dieback and death. Young esca is characterized by brown wood streaking, vascular gummosis, and wood discoloration and may present symptoms in leaves (chlorosis or necrosis) and berries (spotting). White-rot is provoked by wood-rotting basidiomycetes, especially *Fop*, and may or may not be accompanied

by external symptoms in leaves and fruits. Finally, esca proper, includes infection by both basidiomycetes and deuteromycetes associated with esca (*Pch*, *Pal* and *Fop*) and results in the full-scale of internal and external esca symptoms (Graniti *et al.*, 2000; Mugnai *et al.*, 1999).

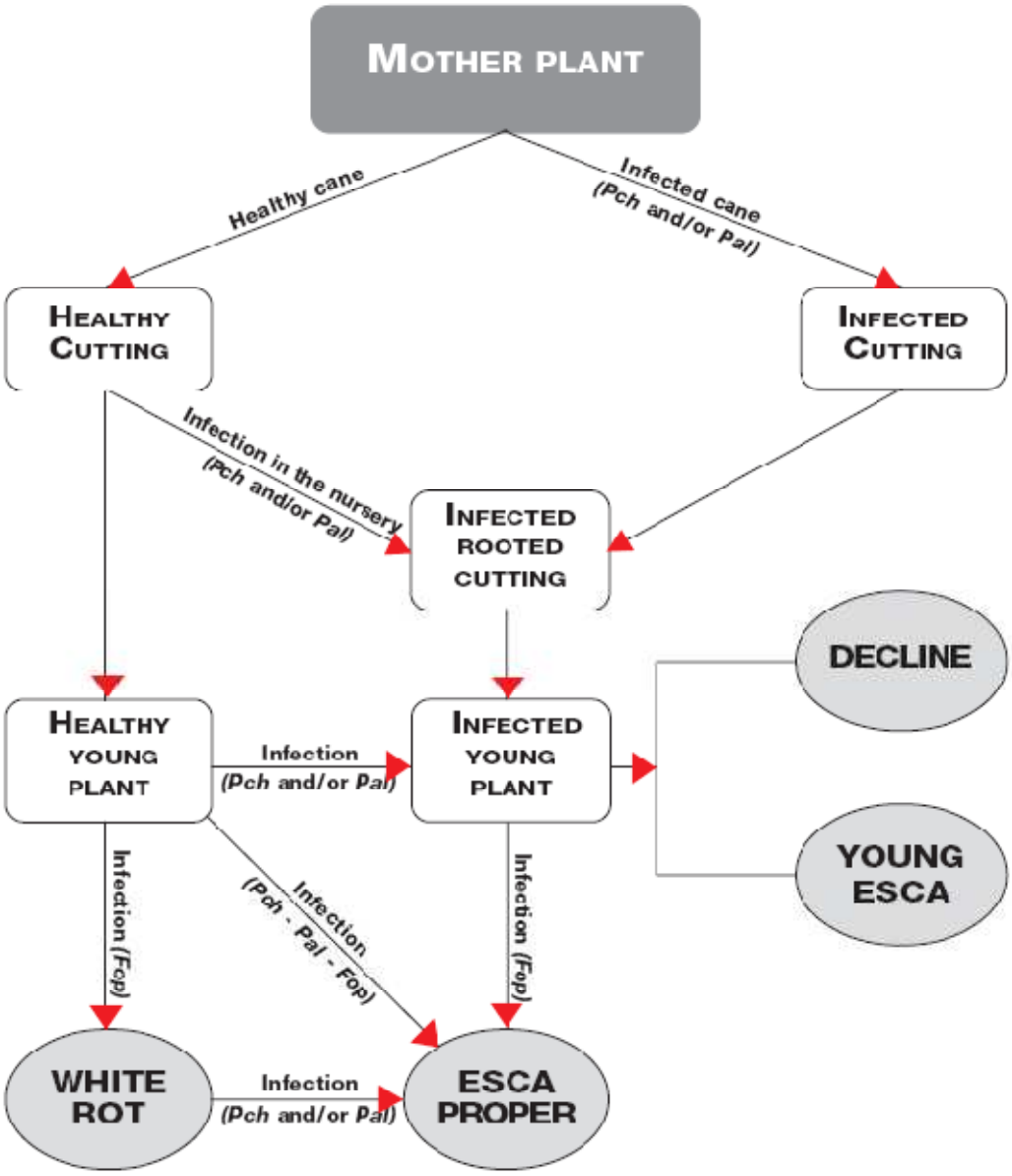


Figure 1.7 – Proposed scheme of the development of esca and related syndromes (from Surico, 2001).

The model considers that *Pch* and/or *Pal* infection may originate in cuttings from infected mother plants, or infection can occur in the nursery. These infected rooted cuttings may originate plants showing young grapevine decline or young esca. The development of either one of these syndromes will depend on host and environmental factors. Healthy young plants originating from uninfected propagating material and treated with proper sanitary care in the nursery may be infected later through pruning wounds. If it becomes infected with *Pch* and/or *Pal*, it can develop young grapevine decline or young esca; if it becomes infected with *Fop* it may develop white-rot; if it becomes infected with the 3 fungi it may develop esca proper. In the same way, white-rotted plants (with *Fop* infection) that become infected with *Pch* and/or *Pal* may develop esca proper, as well as if plants already showing young grapevine decline or young esca (infected with *Pch*/*Pal*) become infected with *Fop* may also develop esca proper (Surico, 2001).

1.2.3 – Grapevine's response to the disease

Until now, the research on esca has been focusing in the isolation and identification of fungi provoking the disease, the mode of spread of these fungi, and on practices that might reduce esca contamination. Few studies were made concerning the response of the plant towards esca. The studies that do exist are mainly related to the production of phytoalexins in esca-affected grapevines.

In 2000, a study detected an increase in *trans*-resveratrol and ε-viniferin in brown-red wood of esca-diseased grapevines when compared to healthy wood, where the compounds were also present but in lower concentrations (Amalfitano *et al.*, 2000). These results suggested that the grapevines initiated a defence response towards esca infection and that resveratrol (and related compounds) may take a part in esca-grapevine interaction.

The activity of resveratrol and pterostilbene against esca-associated fungi (*Pch*, *Pal* and *Fop*) was assayed. Resveratrol revealed to be a poor

fungitoxic against *Pch* and *Pal*, but had some inhibitory effect in the growth of *Fop*, when present in high concentrations. Pterostilbene, on the other hand, proved to have fungitoxic activity against all fungi tested. Interestingly, the addition of phosphorous acid to either of the phytoalexins, increased their fungitoxic potential – phosphorous acid is the resulting product of the rapid degradation of fosetyl-Al (Mazzullu *et al.*, 2000).

More recently, the accumulation of total and recurring phenolics was analysed in *calli* inoculated with esca-associated fungi, as well as in xylem sap and leaves of esca-diseased grapevines (proved to be infected with *Pch*, *Pal* and *Fop*). Total and recurring phenolics were shown to increase in *calli* of an intermediate resistant grapevine variety, while in a susceptible variety the levels of total and recurring phenolics were lower than control. The total phenolic levels were also shown to be higher in xylem sap of diseased vines; however, the levels of recurring phenolics analysed in the sap were higher in healthy than in diseased grapevines. The levels of total and recurring phenolics in leaves of diseased plants were shown to vary during the 4 phenological phases tested (stretched out leaves, fruit setting, cluster closing and bunch ripening). Interestingly, the recurring phenolics isolated in the leaves of infected vines were most of the same phenolics isolated from infected *calli*. Again, like in *calli*, the capacity of producing phenolics seemed to be correlated with lower susceptibility to esca, since the intermediate resistant variety studied produced higher levels of phenolics than the susceptible grapevine variety (Bruno and Sparapano, 2006a; Bruno and Sparapano, 2006b).

1.3 – Aim and outline of the work

As can be concluded from the previously cited works, esca incidence had a huge increase worldwide in the last two decades. Some regions are already severely affected while the disease incidence continues to increase in several countries, including Portugal. Until now, no effective method could be developed to control the esca. This scenario has been leading to important losses in longevity and productivity of grapevines and in wine quality, as well as carrying great economic costs for replanting vineyards.

In RDVV, esca and young grapevine decline has also been leading to important losses in wine production. The disease is widespread all over the region, being present in all varieties of *Vinho Verde* grapevines, and affecting both old and young plants (Chicau *et al.*, 2000).

The aim of this thesis is to contribute to the study of *V. vinifera* defence mechanisms towards esca, using the *Vinho Verde* grapevine varieties Alvarinho and Vinhão. First, a laboratory model using cells suspensions of the esca tolerant grapevine variety Vinhão, is validated to study several plant's defence mechanisms in a rapid and controlled manner. Then, leaves of in field grapevines, of the esca susceptible variety Alvarinho, are analysed in order to evaluate if a defence response is being mounted against esca, even in susceptible plants.

This PhD thesis is organised in 5 chapters. A brief description of each one is given below.

Chapter 1 includes a general introduction concerning RDVV and a review on current knowledge of esca disease.

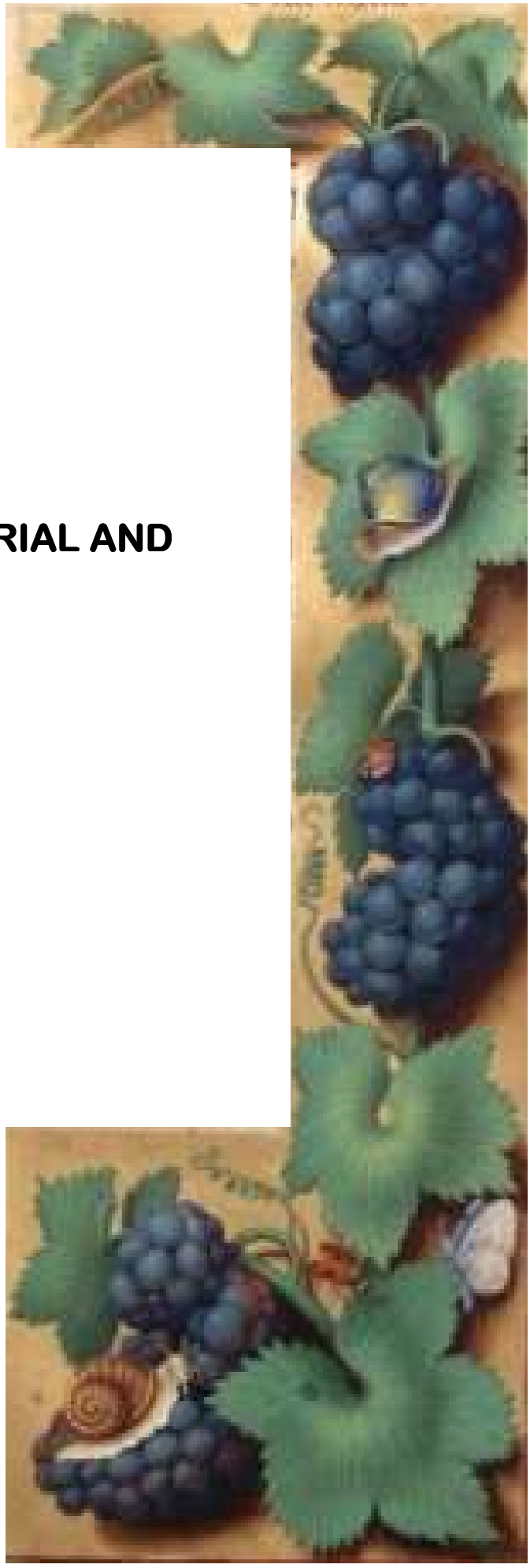
Chapter 2 includes the materials and methods used in the work presented in this thesis.

Chapter 3 includes an introduction on plant defence responses, and describes the establishment of *V. vinifera* cv. Vinhão cell suspension cultures and the results of several defence mechanisms induced by *Pch* extract: production of phenolic compounds and the influence of the phytohormones salicylic acid (SA) and methyl jasmonate (MeJ); the induction of an oxidative burst and the influence of SA and MeJ; the dependence of the oxidative burst on calcium (Ca^{2+}); the contribution of NADPH oxidase, peroxidase and catalase activities to the oxidative burst; and the induction of defence-related gene expression.

Chapter 4 includes a brief introduction on plant metabolomics, and describes the results from the analysis of phenolic and overall metabolites present in leaves of healthy and esca affected grapevines.

Chapter 5 includes the overall conclusions of the work and future perspectives for the continuation of the study.

CHAPTER 2 - MATERIAL AND METHODS



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2.1 – Biological material

2.1.1 – Plant material

2.1.1.1 – V. vinifera *cv. Vinhão* calli *and cell suspension cultures*

2.1.1.1.1 – Establishment and maintenance

V. vinifera cv. Vinhão cuttings collected in May/2003 in Braga (Vinho Verde region in the North of Portugal), were maintained in the lab, in tap water, at 25 ºC and 16h/8h light/dark photoperiod illuminated with fluorescent light bulbs (Osram Fluora) with a photon flux of 30 μmol/m^2s, until growing new leaves.

The leaves were pulverized with 1% fungicide solution (Benlate®) for 1 week before grown leaves were eliminated and stem segments were sectioned leaving the meristem intact. The leaf segments were then surface-sterilized washing 2 times with deionised water followed by washing in a 0.5% Triton-X (Merck KGaA, Darmstadt, Germany) solution for 5 minutes with continuous agitation. Leaf segments were then rinsed 5-6 times with deionised water (until complete removal of detergent) and were placed in a 2% fungicide solution for 2 hours with periodical agitation. Further sterilization steps took place in a sterile vertical flux hood: leaf segments were rinsed one by one in sterile deionised water to remove fungicide and placed in a 1% chlorine solution (prepared from commercial bleach) for 5 minutes with continuous agitation. Then the segments were rinsed 5-6 times in sterile deionised water and placed in solid medium (Table 2.1), and stored at 25 ºC and 16h/8h light/dark photoperiod illuminated with fluorescent light

bulbs (Osram Fluora) with a photon flux of 30 μmol/m^2s, until *calli* were originated.

Table 2.1 – Composition of culture medium used for growing *V. vinifera* cv. Vinhão *calli* and cell suspensions.

Medium components	Reagent	Concentration
Gamborg B5 macronutrients	KNO_3 (Merck KGaA, Darmstadt, Germany)	2.5 g/l
	$(NH_4)_2SO_4$ (Merck KGaA, Darmstadt, Germany)	134 mg/l
	$CaCl_2.2H_2O$ (Merck KGaA, Darmstadt, Germany)	150 mg/l
	$NaH_2PO_4.H_2O$ (Merck KGaA, Darmstadt, Germany)	150 mg/l
	$MgSO_4.7H_2O$ (Merck KGaA, Darmstadt, Germany)	250 mg/l
Murashige and Skoog micronutrients (Duchefa, Haarlem, Netherlands)	$MnSO_4.H_2O$	16.9 mg/l
	$ZnSO_4.7H_2O$	8.6 mg/l
	H_3BO_3	6.2 mg/l
	KI	0.83 mg/l
	$Na_2MoO_4.2H_2O$	0.25 mg/l
	$CuSO_4.5H_2O$	0.025 mg/l
	$CoCl_2.6H_2O$	0.025 mg/l
	$FeSO_4.7H_2O/Na_2.EDTA.2H_2O$	27.8 g/l / 37.2 g/l
Vitamins	Biotin (BDH, Dorset, England)	0.01 mg/l
	Calcium pantothenate (Sigma, St. Louis, USA)	1 mg/l
	Nicotinic acid (Sigma, St. Louis, USA)	1 mg/l
	Thiamine (Sigma, St. Louis, USA)	1 mg/l
	Pyridoxine (BDH, Dorset, England)	1 mg/l
	Myo-inositol (Sigma, St. Louis, USA)	100 mg/l
Hormones	Kinetin (Sigma, St. Louis, USA)	0.2 mg/l
	α-Naphthaleneacetic acid (Sigma, St. Louis, USA)	0.1 mg/l
Carbon source	Sucrose (Panreac, Barcelona, Spain)	20 g/l
Others	Casein hydrolysate (BD Diagnostic Systems, Sparks, USA)	250 mg/l
	Agar (José M. Vaz Pereira, Lisbon, Portugal)	8 g/l
pH was adjusted to 6.0 and medium was autoclave sterilized for 15 minutes (120ºC, 1 atm)		

Calli cultures were maintained in solid medium (Table 2.1), at 25 ºC and 16h/8h light/dark photoperiod illuminated with fluorescent light bulbs (Osram Fluora) with a photon flux of 30 μmol/m^2s. Subculture occurred every 4 weeks.

Cell suspension cultures were initiated by placing chopped 3 weeks old *calli* in 250 ml Erlenmeyer flasks containing 70 ml of liquid medium with the same composition described in Table 2.1, except agar. Cell cultures were maintained in this liquid medium, at 25 ºC, 16h/8h light/dark photoperiod illuminated with fluorescent light bulbs (Osram Fluora) with a photon flux of 30 μmol/m^2s and shaken at 100 rpm. Subculture occurred every 2 weeks by transfer of 10 ml into fresh medium. After 5-7 subcultures, a homogeneous suspension culture was obtained, and was used for elicitation experiments.

2.1.1.1.2 – Elicitation procedures/experiments with inhibitors

Elicitors/inhibitors were added to *V. vinifera* cv. Vinhão cell suspensions in the exponential growth phase, at 5^{th} and/or 6^{th} day after subculture. During the experiments, cells suspensions remained under normal conditions of light, temperature and agitation, and time course samples were collected. For phenolic analysis of grapevine cell suspensions, cells were harvested 24 and/or 72 hours after elicitation, collected by centrifugation and freeze-dried for 48 hours.

For *Pch* elicitation, *Pch* extract was added to cell cultures to a final concentration of 0.5 mg of fungal biomass per ml of cell suspension.

For phytohormones priming/elicitation, MeJ and SA were added to the cell suspensions to a final concentration of 100 μM.

The NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) was added to suspension cultures to 10 μM final concentration.

The Ca^{2+} channels inhibitors nifedipine, ruthenium red and lanthanum (III) chloride ($LaCl_3$) were added to cell suspensions to a final concentration of 100 μM, and the Ca^{2+} chelator ethylene glycol-bis(2-aminoathylether)-N, N, N'; N'-tetraacetic acid (EGTA) was added to 5mM final concentration.

2.1.1.2 – V. vinifera *cv. Alvarinho leaves*

V. vinifera cv. Alvarinho leaves were collected in Brejoeira (Vinho Verde region in the North of Portugal). Ten grapevines were selected and from each one were collected: 2 leaves without symptoms from a cordon without visible foliar symptoms (healthy leaves - hl); 2 leaves without symptoms from a cordon with visible foliar symptoms (apparently healthy leaves - ahl), and 2 leaves showing esca foliar symptoms from the latter cordon (diseased leaves - dl). The leaves were freeze-dried and ground individually.

2.1.2 – Fungus *Pch*

2.1.2.1 – Isolation and maintenance

Pch fungus was isolated from diseased wood of *V. vinifera* cv. Alvarinho collected in Vinho Verde region in the North of Portugal. The fungus was maintained in Petri plates with potato dextrose agar (PDA; Difco), in the dark at 25 ºC. *Pch* was subcultured every 4 weeks by placing a 0.5 cm^2 cube of PDA with grown fungus into fresh medium.

2.1.2.1 – Extract preparation

To obtain fungal biomass for extract preparation a 3 week old *Pch* plate was cut into pieces and these were put into a 1 l Erlenmeyer flask containing 200 ml of fresh Mathur liquid medium (Table 2.2). The Erlenmeyer was placed at 26 ºC and shaken at 250 rpm. After 2 weeks the mycelium was harvested by filtration, freeze-dried for 48 hours and ground with mortar and pestle. A *Pch* suspension was prepared by ressuspending the lyophilized biomass in deionised water (40 mg/ml) and autoclaving for 15 minutes (120 ºC, 1 atm).

Table 2.2 – Mathur liquid medium for growing *Pch* biomass.

Reagent	Concentration
Yeast extract (Cultimed)	1 g/l
Bacto peptone (Difco)	1 g/l
$MgSO_4.7H_2O$ (Merck KGaA, Darmstadt, Germany)	2.5 g/l
KH_2PO_4 (Merck KGaA, Darmstadt, Germany)	2.7 g/l
Sucrose (Panreac, Barcelona, Spain)	10 g/l
pH was adjusted to 5.8 and medium was autoclave sterilized for 15 minutes (120 ºC, 1 atm)	

2.2 – Biochemical methods

2.2.1 – Phenolic compounds analysis

2.2.1.1 – V. vinifera *cv. Vinhão cell suspension cultures*

Freeze-dried biomass of grapevine cv. Vinhão cells (100-200 mg) was extracted in the dark with 5 ml of a 70% methanol (Merck KGaA, Darmstadt, Germany) solution acidified with 0.5% formic acid (Merck KGaA, Darmstadt, Germany), for 24 hours. The liquid phase was syringe filtered and samples were stored at 4 ºC until HPLC analysis.

The methanolic extracts were directly subjected to high performance liquid chromatography-diode array detection (HPLC-DAD) analysis. Chromatographic separation was carried on a chromatographic Beckman Gold system equipped with a RP C18 column - 25x0.4 mm; particle size 5 μm – (Merck KGaA, Darmstadt, Germany), using water/formic acid (99:1) and methanol as the mobile phases. The elution gradient utilized was 5% of methanol at time 0 minutes, and 95% methanol at time 45 minutes. Chromatograms were recorded at 306 and 520 nm. Quantification of compounds was made by the external standard method. Stilbenes type compounds were quantified at 306 nm, as resveratrol (Extrasynthese, Geney, France) equivalents, while anthocyanins were quantified at 520 nm as cyanidin-3-rutenoside (Extrasynthese, Geney, France) equivalents.

Phenolic identification was also performed by HPLC-mass spectrometry (HPLC-MS-MS). Chromatographic separation was carried out on a RP C18 column - 25x0.4 mm; particle size 5 μm – (Merck KGaA, Darmstadt,

Germany), using water/formic acid (99:1) and methanol as the mobile phases. Elution was performed as described above. The HPLC system was an Agilent HPLC 1100 instrument series equipped with an Agilent DAD detector - G1315B – (Agilent Technologies, Germany) and mass detector in series, controlled by software from Agilent Technologies. The mass detector was an ion-trap mass spectrometer - G2445A – (Agilent Technologies, Germany) equipped with an electrospray ionization (ESI) system. The heated capillary and voltage were maintained at 350 ºC and 4 kV, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 l/min, respectively. Mass scan (MS) and daughter (MS–MS) spectra were measured from 100 au to m/z 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired, both in the negative and positive modes.

2.2.1.2 – V. vinifera *cv. Alvarinho leaves*

The phenolic content of healthy, apparently healthy and diseased leaves (13 of each, corresponding to 1 leaf from each of the 10 grapevines selected plus 3 randomly chosen) was analysed.

Freeze-dried biomass of grapevine cv. Alvarinho leaves (100-200 mg) was extracted in the dark with 5 ml of a 70% methanol (Merck KGaA, Darmstadt, Germany) solution acidified with 0.5% formic acid (Merck KGaA, Darmstadt, Germany), for 24 hours. The liquid phase was syringe filtered and samples were stored at 4 ºC until HPLC analysis.

Samples were analyzed by HPLC-DAD-MS: compounds separation was performed in a chromatographic Beckman Gold system, equipped with a RP C18 column - 25x0.4 mm; particle size 5 μm – (Merck KGaA, Darmstadt, Germany), using water/formic acid (99:1) and methanol as the mobile phases. The elution gradient utilized was the same described in 2.2.1.1. Chromatograms were recorded at 306 nm. Quantification of compounds was made by the external standard method. Phenolic compounds were

quantified at 306 nm, as resveratrol (Extrasynthese, Geney, France) equivalents.

Also, samples were analyzed by LC-MS; Agilent 1100 LC/MSD, negative ion mode; scan range 200-1500 amu, as described above.

2.2.2 – ROS determination

The probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene - Orlando, USA) was used to detect total intracellular ROS production in *V. vinifera* cv. Vinhão suspension cells, elicited with *Pch* extract, elicited/primed with phytohormones, in the presence of NADPH oxidase inhibitor and in the presence of Ca^{2+} chelator and Ca^{2+} channels inhibitors (described in 2.1.1.1.2). The procedure was based in previous description (Parsons *et al.*, 1999). It is thought that H_2DCF-DA enters the cells, and is deacetylated to 2',7'-dichlorodihydrofluorescein (H_2DCF) and rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF), in the presence of ROS (LeBel *et al.*, 1992).

After elicitation of cell cultures, time course samples (1 ml) were taken. These samples were added to 10 µl of 200 µM H_2DCFDA solution (to 2 µM final concentration) and quickly mixed in the vortex. Samples were incubated in the dark, at room temperature, for 15 minutes. The samples were quick-spinned (to sediment cells) and 500 µl of supernatant was collected and diluted with 2.5 ml of ultrapure water. Fluorescence was measured at room temperature in a LS50 Luminescence Spectrometer (Perkin Elmer, Buckinghamshire, England), with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Fluorescence data was analysed according to previously described (Wang and Joseph, 1999). Fluorescence percentage increase was calculated using the formula $\%\ fluorescence\ increase = \frac{(Fx-F0)}{F0} \times 100$; where F*x* represents fluorencence at *x* minutes and F0 represents fluorescence at 0 minutes.

2.2.3 – Enzymatic activities

2.2.3.1 – Catalase

Catalase activity of *V. vinifera* cv. Vinhão cell suspensions after elicitation with *Pch* extract (see 2.1.1.1.2) was determined as previously described (Conrath *et al.*, 1995).

Time course 250 µl samples were taken and diluted 1:5 in fresh liquid culture media (table 2.1), and placed in the chamber of an oxygen (O_2) electrode (Hansatech, Great Britain). After the establishment of a constant baseline, catalase activity was measured by continuous recording for 1-2 minutes the rate of hydrogen peroxide (H_2O_2)-dependent O_2 production after addition of 10µl of a freshly prepared 2M H_2O_2 (Merck KGaA, Darmstadt, Germany) solution (to 16 mM final concentration).

Rates were obtained calculating the slope of the linear zone recorded using the formula $m = \frac{(y2-y1)}{(x2-x1)}$; where *m* is the slope and (*x*1, *y*1) and (*x*2, *y*2) are coordinates of 2 points in the straight line.

2.2.3.2 – Peroxidase

After elicitation with *Pch* extract (described in 2.1.1.1.2) of *V. vinifera* cv. Vinhão cell suspensions, time course samples (15 ml) were taken. Cells were recovered by centrifugation at 4 ºC and immediately frozen in liquid nitrogen and stored at -80 ºC.

The frozen biomass was reduced to powder with mortar and pestle in the presence of liquid nitrogen. Approximately 2.5 ml of frozen biomass powder was placed in a 15 ml tube on ice, 4 ml of extraction buffer (table 2.3) was added, and the mixture was allowed to stand at 4 ºC for 40 minutes, with slight agitation.

The homogenate was then filtered through 4 layers of gauze, collected in a centrifuge tube, and centrifuged at 13000 *g*, at 4 ºC, for 25 minutes. Supernatant was recovered and purified through Sephadex G25 PD10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), according to manufacturer instructions. Briefly, the column was equilibrated with 25 ml of buffer (extraction buffer was used) discarding the flow-through; 2.5 ml of sample was added to the column and flow-through was discarded; finally, protein extract was recovered eluting with 3.5 ml of extraction buffer. Extract was stored at -80 ºC until enzyme activity determination.

Table 2.3 – Extraction buffer used to prepare protein extracts for peroxidase activity determination.

Reagent	Concentration
Tris-HCl pH 7.5 (USB, Cleveland Ohio, USA and Panreac, Barcelona, Spain; respectively)	50 mM
Ascorbic acid (Sigma, St. Louis, USA)	6 mM
KCl (Sigma, St. Louis, USA)	1 M
$MgCl_2$ (Sigma, St. Louis, USA)	1M
$CaCl_2$ (Sigma, St. Louis, USA)	1 M
$MnCl_2$ (Sigma, St. Louis, USA)	1 M
NaCl (Sigma, St. Louis, USA)	1 M
Extraction buffer was freshly prepared before use	

Coomassie Blue method (Bradford, 1976) was used to quantify protein in the extract. A reaction mixture of 100 µl of an appropriate dilution of the protein extract and 900 µl of Coomassie Blue reagent (table 2.4) was prepared and incubated for 10 minutes in the dark, at room temperature. Samples were placed in a quartz cuvette and absorbance was read at 595 nm in a Cary 1E/3E UV-Vis Spectrophotometer (Varian, Australia). Protein was quantifyed using a Bovine Serum Albumine (Sigma, St. Louis, USA) standard curve (0.5 – 15 µg).

Table 2.4 – Coomassie Blue reagent.

Reagent	Concentration
Coomassie® Brilliant Blue G 250 (Merck KGaA, Darmstadt, Germany)	0.06 % (w/v)
Perchloric acid (Sigma, St. Louis, USA)	1.9 % (v/v)
Solution was filtered through Whatman ® No.1 filter paper and stored in the dark, at room temperature	

Peroxidase activity was determined as previously described, based on the H_2O_2-dependent oxidation of 4-methoxy-α-naphthol (4-MN) to yield a deep-blue product (Ferrer *et al.*, 1990). The kinetic assay was performed in a Cary 1E/3E UV-Vis Spectrophotometer (Varian, Australia), at room temperature, on a 1 ml assay volume. In a spectrophotometer cuvette, 10 µl of protein extract was added to 947 µl of a 100 mM Tris-Acetate pH6.0 (USB, Cleveland Ohio, USA and Merck, Darmstadt, Germany; respectively) solution and 10 µl of a 100 mM 4-MN (Sigma, St. Louis, USA) solution prepared in ethylene glycol monomethyl ether (Sigma, St. Louis, USA). The mixture was mixed and absorbance was read at 593 nm. After, 33 µl of a 10 mM H_2O_2 (Merck KGaA, Darmstadt, Germany) solution was added, mixed and the absorbance read continuously for 2 minutes.

Peroxidase activity of the protein extract was calculated in nkatal/ml*ng protein using the formula $Activity = \frac{\frac{Absorbance}{minute}}{1.26} \times \frac{1000}{extract\ dilution\ factor}$; and then corrected for the amount of protein present in the extract. The extinction coefficient (ε) of 4-MN oxidation product is ε_{593} = 21 000/M cm.

2.2.3.3 – NADPH oxidase

After elicitation with *Pch* extract and/or DPI (described in 2.1.1.1.2) of *V. vinifera* cv. Vinhão cell suspensions, time course samples (15 ml) were taken. Cells were recovered by centrifugation at 4 ºC and immediately frozen in liquid nitrogen and stored at -80 ºC.

The frozen biomass was reduced to powder with mortar and pestle in the presence of liquid nitrogen. A plasma membrane extract was prepared based in a previously described method (Widell *et al.*, 1982). Approximately

2.5 ml of frozen biomass powder was placed in a centrifuge tube on ice and 5 ml of extraction buffer (table 2.5) was added. The biomass powder was allowed to thaw in the buffer for 10 minutes. After centrifugation at 10000 *g*, at 4 ºC, for 15 minutes, the supernatant was recovered to centrifuge tubes and centrifugated at 30000 *g*, at 4 ºC, for 60 minutes. The supernatant was discarded, and the pellet ressuspended in ressuspention buffer (table 2.6).

Table 2.5 – Extraction buffer used to prepare plasma membrane extracts for NADPH oxidase activity determination.

Reagent	Concentration
Tris-HCl pH 7.5 (USB, Cleveland Ohio, USA and Panreac, Barcelona, Spain; respectively)	10 mM
Sucrose (Panreac, Barcelona, Spain)	250 mM
EDTA (Sigma, St. Louis, USA)	1 mM
Extraction buffer was freshly prepared before use	

Table 2.6 – Ressuspention buffer used to prepare plasma membrane extracts for NADPH oxidase activity determination.

Reagent	Concentration
K-phosphate pH 7.8	5 mM
Sucrose (Panreac, Barcelona, Spain)	250 mM
Ressuspention buffer was freshly prepared before use	

The homogenate was purified through Sephadex G25 PD10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), according to manufacturer instructions. Briefly, the column was equilibrated with 25 ml of buffer (resuspention buffer was used) discarding the flow-through; 2.5 ml of sample was added to the column and flow-through was discarded; finally, the plasma membranes extract was recovered eluting with 3.5 ml of ressuspention buffer. Extract was stored at -80 ºC until enzyme activity determination.

Protein was quantified using Coomassie Blue method, as described in 2.2.3.2.

NADPH oxidase activity was determined as previously described, based on the rapid conversion of nitro blue tetrazolium (NBT) to monoformazan by 2 molecules of O_2^- (Van Gestelen *et al.*, 1997). The kinetic assay was performed in a Cary 1E/3E UV-Vis Spectrophotometer (Varian, Australia), at room temperature, on a 1 ml assay volume. The amount of extract necessary to have 10-50 μg of protein was placed in a spectrophotometer cuvette and reaction buffer (table 2.7) was added until 1 ml. The mixture was mixed and absorbance was read at 530 nm. After, 50 μl of a 2 mM NADPH (Merck KGaA, Darmstadt, Germany) solution was added mixed and the absorbance was read continuously for 2 minutes. Then, 10.6 μl of a 1 mg/ml super oxide dismutase (SOD; Merck KGaA, Darmstadt, Germany) solution was added (to a final concentration 50 u/ml in the reaction), mixed and the absorbance was read continuously for 2 minutes.

Table 2.7 – Reaction buffer used for NADPH oxidase activity determination.

Reagent	Concentration
Tris-HCl pH 7.5 (USB, Cleveland Ohio, USA and Panreac, Barcelona, Spain; respectively)	20 mM
Sucrose (Panreac, Barcelona, Spain)	250 mM
NBT (Merck KGaA, Darmstadt, Germany)	0.1 mM
Reaction buffer was freshly prepared before use	

NADPH oxidase activity was calculated as the difference in NBT reduction rate in the presence and absence of SOD, and expressed as nkatal/ml*ng protein. It was used the formula $Activity = \frac{\frac{Absorbance}{minute}}{76.8} \times \frac{1000}{extract\ dilution\ factor}$; and then corrected for the amount of protein present in the extract. The extinction coefficient of monoformazan is ε_{530} = 12 800/M cm.

2.2.4 – Nuclear magnetic resonance (NMR) analysis

The overall metabolic content of healthy and diseased leaves (10 of each, corresponding to 2 leaves from each of 5 grapevines selected in the field) was analysed.

Freeze-dried biomass of grapevine cv. Alvarinho leaves (100 mg) was added to 750 µl of methanol-d_4 (Sigma, St. Louis, USA) plus 750 µl of KH_2PO_4 (Merck KGaA, Darmstadt, Germany) buffer (pH 6.0) in D_2O (Sigma, St. Louis, USA) containing 0.1 % (w/w) trimethyl silane propionic acid sodium salt (TSP; Sigma, St. Louis, USA), briefly vortexed, sonicated for 20 minutes and incubated overnight at 4 ºC. Then, samples were centrifugated to sediment the biomass and 800 µl of extract was recovered and stored at 4 ºC until NMR spectroscopy analysis.

All proton (^{1}H) NMR spectra were recorded at 300K on a Bruker DRX-500 spectrometer (University of Aveiro) operating at a frequency of 500.13 MHz. Standard 1D ^{1}H spectra were acquired using the "noesypr1d" (RD–90º–t_1–90º–t_m–90º–acquire) pulse sequence using a 100 ms mixing time (t_m) and a fixed 4 µs t_1 delay were used and water presaturation was performed during the relaxation delay and mixing time. The acquired spectra consisted of 128 transients, with 64k complex data points, 10330.58 Hz spectral width (SW) and 1.5 s relaxation delay. Prior to Fourier transformation (FT), the free induction decays (FIDs) were zero-filled to 64k points and multiplied by an exponential line-broadening function of 0.3 Hz. The 1D spectra were manually phased, baseline corrected and the chemical shifts referenced internally to the α-glucose H1 resonance at 5.23 ppm. Total correlation spectroscopy (TOCSY) ^{1}H-^{1}H spectra were recorded in phase sensitive mode using States-TPPI (time proportional phase incrementation) detection in t_1 with a MLEV17 spin lock pulse sequence (Bax and Davis, 1985). 32 FIDs with 8k complex data points per increment and a total 350 increments were acquired with SW 8002.13 Hz and 8012.82 Hz (for F1 and F2 dimensions respectively), 90 ms mixing time and 1.4 s relaxation delay.

Heteronuclear single quantum coherence (HSQC) ^{1}H-^{13}C spectra was acquired with inverse detection, ^{13}C decoupling during acquisition, recording 64 FIDs with 4k complex data points per increment to a total of 300 increments, with SW 8012.82 and 25153.81 Hz for ^{1}H and ^{13}C dimensions, respectively and 1.5 s relaxation delay. Both 2D spectra were manually phased in both dimensions, after Fourier transformation and zero filling, baseline corrected and referenced internally to the α-glucose H1 resonance at 5.23 ppm in ^{1}H dimension and to the carbon methyl from TSP at 0 ppm in the ^{13}C dimension.

2.2.5 – Statistical analysis

For HPLC assays of *V. vinifera* cv. Vinhão cell suspensions two independent experiments were performed with three independent replicates each. Average and standard deviations were calculated, followed by single factor analysis of variance (ANOVA) and Dunnett's post-test for comparison of two or more groups with control.

For enzyme assays in *V. vinifera* cv. Vinhão cell suspensions two independent experiments with triplicates were performed. Average and standard deviations were calculated, followed by independent *t*-test for comparison of just two groups (treatment with control) or by single factor ANOVA and Dunnett's post-test for comparison of two or more groups with control.

For ROS determination assays in *V. vinifera* cv. Vinhão cell suspensions three independent experiments with duplicates were performed. Treatment averages were calculated following calculation of % of fluorescence increase as described in 2.2.2.

For HPLC results of *V. vinifera* cv. Alvarinho leaves, average and standard deviations were calculated, followed by single factor ANOVA and Tukey's post-test for comparison of every group. Also, peak areas (306nm) were

corrected by the amount of biomass extracted of the corresponding sample. Peaks were manually aligned one by one based on relative retention time and UV spectra. Data was imported to the statistical program SIMCA-P (Umetrics, Umea, Sweden) and a principal components analysis (PCA) was applied after unit variance scaling of the data.

From NMR results of *V. vinifera* cv. Alvarinho leaves, data matrixes of integrated regions of variable width, of three regions (aliphatic, sugar and aromatic), were subjected to PCA using the software Cats97 developed by *Universidade de Aveiro* and *Institute National Agronomique Paris-Grignon* (Barros, 1999).

2.3 – Molecular biology methods

2.3.1 – RNA extraction

After elicitation with *Pch* extract (described in 2.1.1.1.2) of *V. vinifera* cv. Vinhão cell suspensions, time course samples (15 ml) were taken. Cells were recovered by centrifugation at 4 ºC and immediately frozen in liquid nitrogen and stored at -80 ºC.

The frozen biomass was ground to powder with mortar and pestle in the presence of liquid nitrogen.

Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) - which provides enrichment in mRNA in the final product – following supplier's instructions with minor alterations. Briefly, 2 % (w/v) polyvinylpyrrolidone (PVP; Sigma, St. Louis, USA) was added to supplied buffer RTL. Then, 450 µl of this solution (buffer RLT + PVP) was added to 100 mg of frozen biomass powder, and vortexed vigorously. The lysate was transferred to a supplied QIAshredder spin column placed in a collection tube, and centrifuged for 2 minutes at maximum speed. The supernatant of the flow-through was recovered to a new microcentrifuge tube and 0.5 volume of absolute ethanol (Merck KGaA, Darmstadt, Germany) was added and mixed by pipetting. The sample was then transferred to a supplied RNeasy spin column placed in a collection tube and centrifuged at 8000 g for 15 seconds; the flow-through was discarded. After, 700 µl of supplied buffer RW1 was added to the RNeasy spin column that was again centrifuged at 8000 g for 15 seconds; the flow-through was discarded. This

was followed by addition of 500 µl of supplied buffer RPE to the RNeasy spin column that once more was centrifuged at 8000 g for 15 seconds and the flow-through was discarded. Another 500 µl of supplied buffer RPE were added to RNeasy spin column followed by a 2 minutes centrifugation at 8000 g. The RNeasy spin column was recovered and placed in a new collection tube. RNA was eluted from the column adding 40 µl of RNase-free water and centrifuging for 1 minute at 8000 g. RNA was stored at -80 ºC until further procedures.

To ensure that RNA extraction process was successful, quantity and quality of RNA were determined spectrophotometrically in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and considering that 1 unit of absorbance at 260 nm corresponded to 40 ng/µl of RNA. Also, it was considered that a ratio of absorbance at 260 and 230 nm higher than 2.0 indicated low polysaccharide levels and the ratio of absorbance at 260 and 280 nm should be between 1.8 and 2.1 to indicate pure RNA (no/low protein or phenolics contamination).

2.3.2 – cDNA synthesis

cDNA was synthesized from extracted RNA using the First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania), according to supplier's instructions. Briefly, a reaction mixture containing 10 µl of extracted RNA (± 1-2 µg), 1 µl of supplied oligo$(dT)_{18}$ primer and 1 µl of RNase-free water was prepared on ice, mixed and quick-spinned, and incubated at 70 ºC for 5 minutes. The mixture was then placed on ice and 4 µl of supplied reaction buffer, 1 µl of supplied RiboLock™ ribonuclease inhibitor and 2 µl of supplied dNTP mix were added; after gentle mixing and quick spin the mixture was incubated at 37 ºC for 5 minutes. Then 1 µl of supplied M-MuLV reverse transcriptase was added and the mixture incubated at 37 ºC for 60 minutes. The reaction was stopped by heating at 70 ºC for 10 minutes, and chilled on ice. cDNA was stored at -80 ºC until further procedures.

To ensure that cDNA synthesis process was successful, quantity and quality of cDNA were determined spectrophotometrically in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and considering that 1 unit of absorbance at 260 nm corresponded to 50 ng/µl of DNA. Also, it was considered that a ratio of absorbance at 260 and 230 nm higher than 2.0 indicated low polysaccharide levels and the ratio of absorbance at 260 and 280 nm should be between 1.8 and 2.1 to indicate pure DNA (no/low protein or phenolics contamination).

2.3.3 – Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

After elicitation with *Pch* extract (described in 2.1.1.1.2) of *V. vinifera* cv. Vinhão cell suspensions, the expression levels of genes encoding pathogenesis-related proteins (class 6 and class 10 pathogenesis-related (PR) proteins, β-1,3-glucanase, and class III chitinase) and genes involved in the octadecanoid (lipoxigenase) and phenylpropanoid (phenylalanine ammonia lyase and stilbene synthase) pathways were monitored by semi-quantitative RT-PCR, at 3, 12, 24 and 48 hours after elicitation. Actin gene was used as internal control.

2.3.3.1 – Primers

Primer sequences of actin (ACT), phenylalanine ammonia lyase (PAL), stylbene synthase (STSY), β-1,3-glucanase (GLUC) and class 10 PR protein (PR-10) used, were previously described (Bonomelli *et al.*, 2004). Lipoxigenase (LOX), class III chitinase (CH3) and class 6 PR protein (PR-6) rimer sequences were designed based on the sequence information from the GenBank NCBI database (www.ncbi.nlm.nih.gov) using Primer3 (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primer pairs were ordered from STAB Vida (Oeiras, Portugal). A list of primer pairs used is given in table 2.8 and range from 19 to 22 bp in length.

Table 2.8 – Primers used for semi-quantitative RT-PCR.

Gene	Accession number	Primer	Sequence (5'-3')	Amplicon size (bp)
ACT	AF369524	Sense	AGC TGG AAA CTG CAA AGA GCA G	95
		Antisense	ACA ACG GAA TCT CTC AGC TCC A	
PR-6	AY156047	Sense	AGT TCA GGG AGA GGT TGC TG	165
		Antisense	CCG ATG GTA GGG ACA CTG AT	
PR-10	AJ291705	Sense	ACC ACA CCA AGG GCG ATG TA	93
		Antisense	CGT AGG CTT CGA TAG CCT TGA A	
GLUC	AF239617	Sense	AAT TTG ATC CGC CAC GTC AA	101
		Antisense	TGC GGC TCC TTC TTG TTC TC	
CH3	AJ291507	Sense	AGA TGG CAT AGA CTT TGT CA	415
		Antisense	GTA CTT TGA CCA CAG CAT CA	
LOX	AY159556	Sense	CAT GGA CTC CGC CTA CTG AT	175
		Antisense	GAC CCT CTT CCC TGA CTT CC	
PAL	X75967	Sense	TCC TCC CGG AAA ACA GCT G	101
		Antisense	TCC TCC AAA TGC CTC AAA TCA	
STSY	X76892	Sense	AGG AAG CAG CAT TGA AGG CTC	101
		Antisense	TGC ACC AGG CAT TTC TAC ACC	

2.3.3.2 – Optimization conditions

Initially, the amplification mixture was defined to be the one presented in table 2.9.

Table 2.9 – Composition of reaction mixture used in semi-quantitative RT-PCR.

Reagent	Concentration
Green Flexi Reaction Buffer 5X (Promega Corporation, Madison, USA)	1 x
$MgCl_2$ 25mM (Promega Corporation, Madison, USA)	2.5 mM
Primer sense 10µM	1 µM
Primer antisense 10µM	1 µM
dNTPs 10mM (Fermentas UAB, Vilnius, Lithuania)	0.8 mM
GoTaq® Flexi DNA Polymerase 5u/µl (Promega Corporation, Madison, USA)	5 u
cDNA	2 µl
DNase-free water until 20 µl	

The annealing temperature was optimized in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, USA) using 40 amplification cycles and a temperature gradient between 35 and 60 ºC (all other conditions being the ones described in table 2.10). PCR products were electrophoresed, alongside with a GeneRuler™ 50 bp DNA Ladder (Fermentas UAB, Vilnius, Lithuania), through a 3 % (w/v) agarose (Sigma, St. Louis, USA) gel stained with SYBR Safe™ (Molecular Probes, Eugene - Orlando, USA). Images were captured using a ChemiDoc XRS (Bio-Rad Laboratories, USA). Temperature 60 ºC was selected for all genes in study to be the optimum temperature, that originates a specific PCR product.

After, cycle number was optimized for each gene in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, USA) using a cycle gradient between 26 and 44 cycles in the amplification phase (all other conditions being the ones described in table 2.10). PCR products were electrophoresed, alongside with a GeneRuler™ 50 bp DNA Ladder (Fermentas UAB, Vilnius, Lithuania), through a 3 % (w/v) agarose (Sigma, St. Louis, USA) gel stained with SYBR Safe™ (Molecular Probes, Eugene - Orlando, USA). Images were captured using a ChemiDoc XRS (Bio-Rad Laboratories, USA) and band intensities determined with Quantity One® Software (Bio-Rad Laboratories, USA). The amount of PCR product over the assayed range of cycles was examined, and an appropriate cycle number falling in the linear range was chosen for each gene. The number of cycles found to be appropriate for STSY, LOX, GLUC and CH3 were 34 cycles and 30 cycles for PAL, PR-6 and PR-10. The amplification of ACT was well within the linear amplification phase at both 30 and 34 cycles.

All optimizations were made using pooled cDNA of all samples.

2.3.3.3 – Semi-quantitative RT-PCR

PCR reactions were carried in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, USA) using the amplification mixture detailed in table 2.9 and using the PCR program described in table 2.10.

Table 2.10 – PCR program used to analyse gene expression.

Temperature (ºC)	Time (minutes)	Cycles
95	01:30	1 x
95ºC	00:45	
60ºC	01:00	*
72ºC	01:00	
72ºC	05:00	1 x
4ºC	∞	

* 30 for PAL, PR-6 and PR-10 / 34 for STSY, LOX, GLUC, CH3

PCR products were electrophoresed, alongside with a GeneRuler™ 50 bp DNA Ladder (Fermentas UAB, Vilnius, Lithuania), through a 3 % (w/v) agarose (Sigma, St. Louis, USA) gel stained with SYBR Safe™ (Molecular Probes, Eugene, USA). Images were captured using a ChemiDoc XRS (Bio-Rad Laboratories, USA) and band intensities determined with Quantity One® Software (Bio-Rad Laboratories, USA).

2.3.4 – Results treatment

For gene expression assays in *V. vinifera* cv. Vinhão cell suspensions two independent experiments were performed. All samples were normalized by corresponding actin gene expression. Control samples of each time were defined as 1x expression level.

CHAPTER 3 - INTERACTION *Vitis vinifera* cv. Vinhão–*Phaeomoniella chlamydospora*

In the previous page: illumination by Jean Bourdichon, from *Horae ad usum romanum – Grandes heurs d'Anne de Bretagne* (1503-1508), Tours – France; Bibliothèque nationale de France, Département des Manuscrits, Division occidentale (cote Latin 9474, folio 156). Accessed from Mandragore, base des manuscrits enluminés de la Bibliothèque nationale de France (http://mandragore.bnf.fr/html/accueil.html) on 12th January 2009.

3.1 - Introduction

Plants must be able to live in a challenging environment with both biotic and abiotic stressors acting solely or simultaneously. Everywhere there is a myriad of potential pathogens like virus, fungi, bacteria, nematodes, insects and other herbivores ready to attack the plants for nourishment or to use them to complete their life cycles. Because plants are not able to move, they cannot escape predators or change places to avoid abiotic stressors such as high temperatures or drought. So, plants evolved other types of defence mechanisms that enable them to recognize an attack and respond accordingly (Dangl and Jones, 2001). These defence mechanisms rely in an incredible metabolic plasticity that allows plants to allocate resources from growth and reproduction to defence. The process involves complex signalling networks activated by abiotic and biotic elicitors and followed by extensive crosstalk between signalling pathways, which can result in resistance to subsequent challenges (Bostock, 2005).

Plant defence mechanisms are so successful that great part of plant-microbe interactions in nature do not result in disease. Plants accomplish this using two defence strategies: pre-formed defences and induced defences (Iriti and Faoro, 2007; Veronese *et al.*, 2003).

The pre-formed defences are physical and chemical structures that function as barriers obstructing pathogen penetration. These defences include cell walls, cuticles and suberized tissues that difficult pathogen ingression due to their rigidity. Also, a wide variety of chemical barriers such as terpenoids, hydroxamic acids, cyanogenic glucosides, phenolic compounds and peptides (e.g. defensins), that are embedded in or covering the rigid

structures, help to protect against microorganisms, insects or herbivores since they have a broad antibiotic activity and unpleasant physiological effects on herbivores (Iriti and Faoro, 2007; Menezes and Jared, 2002).

However, if pathogens overcome this first line defences, the plant activates induced resistance mechanisms, resulting in local responses that control infection and sometimes in systemic activation of defence responses leading to systemic acquired resistance (SAR), which provides an integrated and long-lasting immunity of the whole plant against not only the pathogen that caused the primary infection, but to a broader range of stressors (Menezes and Jared, 2002). Generally, resistant and susceptible plants utilize similar defence mechanisms, however it is the fast and intense activation of these induced responses in resistant plants that results in incompatible interactions (no disease), while the slower activation of induced resistance mechanisms in susceptible plants results in compatible interactions. Normally, incompatible and compatible interactions can be distinguished by the presence or absence of hypersensitive response (HR), respectively. The rapid and localised cell death characteristic of HR restricts and controls the infection and seems to be necessary for the establishment of SAR (Walling, 2000).

The activation of induced responses relies on the ability of the plant to discriminate between self and non-self. Once the pathogens defeat first line defences and gain access to the plant's plasma membrane, or even the interior of the cells, several microbe-associated products (elicitors) are recognized by the plant and induced defence mechanisms are activated (Nürnberger *et al.*, 2004).

These elicitors can be general or specific, originating what is called non-host and host resistance, respectively. Non-host resistance is the most common form of resistance and is defined as *the resistance shown by an entire plant species to a specific parasite or pathogen*. Host resistance, on the other hand, *is expressed by plant genotypes within an otherwise susceptible host species* and *is usually parasite-specific* (Heath, 2000).

Host resistance is now thought to be the result of evolution of host-microbe interactions, taking the non-host resistance one step further. Once receptors in the extracellular surface (or inside the cell) recognize general pathogen-associated molecular patterns (PAMPs), induced defence mechanisms are triggered. However, some pathogens have evolved means to suppress triggered defence mechanisms by interfering with PAMPs recognition or by secreting effector molecules that alter resistance signalling inside the plant cell. In response, plants developed more specialized detection mechanisms that can trigger induced defences (Chrisholm *et al.*, 2006). As a result, host resistance is mostly explained by the gene-for-gene hypothesis, in which pathogen avirulence genes (*Avr*) have complementary plant resistance genes (*R*). The perception of *Avr* gene products by R proteins activates plant induced defence responses. So, *Avr* gene products act as specific elicitors (Nürnberger and Brunner, 2002).

The nature of elicitors (general and specific) capable of triggering induced defence mechanisms is very diverse: includes microorganisms' structural components (e.g. chitin) and secreted peptides, proteins and toxins, as well as materials of plant origin such as degraded plant polymers (resulting, for example, by the degradation of plant cell walls by fungal enzymes) or even alterations on cell's carbon, nitrogen and hormone status caused by pathogen feeding (Ebel and Mithöfer, 1998; Hammond-Kossack and Parker, 2003).

The signalling pathways triggered upon recognition of general or specific elicitors are basically the same (Glazebrook, 2005; Nürnberger *et al.*, 2004; Veronese *et al.*, 2003). Several early events start to occur sequentially, amplifying the signal and culminating in the synthesis of defensive molecules both locally and systemically. Although the order of these early events may vary among plant species and/or according to the elicitor that triggers the response, the sequence of events can be ordered as described in Figure 3.1: alteration of phosphorilation status of elicitor receptor in plasma membrane or cytosol and other proteins; calcium influx leading to a spike in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$); membrane depolarization; ion fluxes chlorine (Cl^-) and potassium (K^+) efflux together with proton (H^+) influx;

cytoplasmic acidification; stimulation of mitogen-activated protein kinases (MAPKs); nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation leading to reactive oxygen species (ROS) production; early defence gene expression, ethylene and jasmonate production; late defence gene expression and production of secondary metabolites (Zhao *et al.*, 2005a).

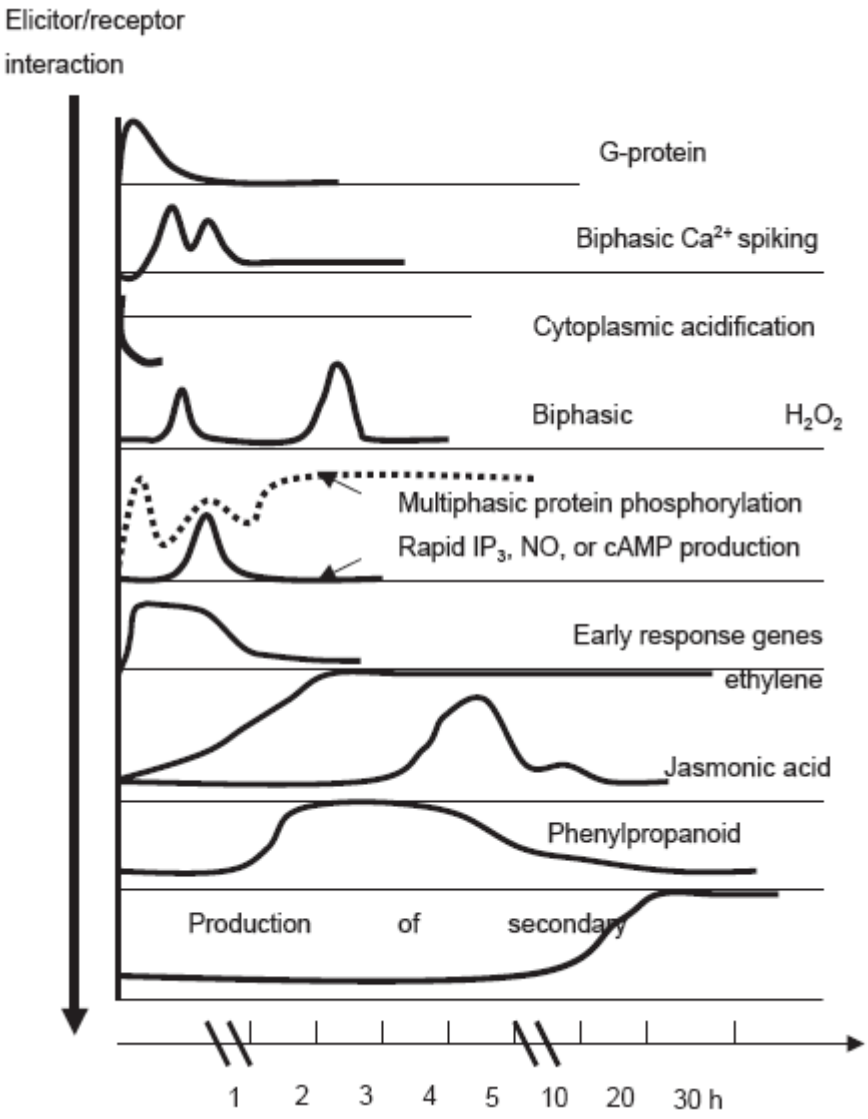

Figure 3.1 – *Schematic illustration of the sequential defence reactions in plants induced by elicitors. In many particular cases, only some of these events were detected* (from Zhao *et al.*, 2005a).

3.1.1 – Ion fluxes and Ca^{2+}

Ion fluxes are generally the earliest responses following elicitor perception, namely K^+/H^+ exchange, Ca^{2+} influx and Cl^- efflux (Zhao *et al.*, 2005a).

Particularly, Ca^{2+} is now a well established second messenger and is considered a converging point of several signalling pathways. The location, duration, amplitude and type of oscillation (single or repeated) of $[Ca^{2+}]_{cyt}$ changes (called Ca^{2+} signature) seems to be elicitor specific and thus leads to a specific and appropriated response to the stimulus (Plieth, 2001; Trewavas and Malhó, 1998).

Both extracellular and internal Ca^{2+} stores are used to increase $[Ca^{2+}]_{cyt}$ in defence responses (Lecourieux *et al.*, 2002). The increase in $[Ca^{2+}]_{cyt}$ activates several intracellular processes directly or interacting with Ca^{2+}-binding proteins (e.g. calmodulin (CaM) and Ca^{2+}-dependent protein kinases (CDPKs)), and can also stimulate downstream events that will amplify Ca^{2+} signalling. Once activated, these Ca^{2+} sensors will induce a series of other downstream processes such as activation of MAPKs cascades (shown to be associated with plant defences induction), activation of NADPH oxidase (leading to ROS production, that in turn can stimulate Ca^{2+} influx), nitric oxide (NO) production (that can activate Ca^{2+} release from internal stores), ion channel modulation (including Ca^{2+} channels), phospholipase activation (leading to jasmonate synthesis and signalling, as well as to synthesis of other second messengers such as inositol-1,4,5-triphosphate (IP_3) that can activate Ca^{2+} release from internal stores), and also activation of transcription factors (that will regulate defence gene expression of PR proteins and enzymes of the phenylpropanoid pathway leading to the production of phytoalexins) (Lecourieux *et al.*, 2006; White and Broadley, 2003; Zhao *et al.*, 2005a).

Moreover, Ca^{2+} may be involved not only in the early stage of signalling, but also participate in the later stages of defence response in HR (Lecourieux *et al.*, 2006; Pontier *et al.*, 1998)

3.1.2 – Production of ROS

Generation of ROS, particularly superoxide (O_2^-) and hydrogen peroxide (H_2O_2), is another early event well documented upon pathogen attack. Generally, an incompatible plant-microbe interaction will induce a biphasic ROS accumulation with a rapid and small first ROS accumulation followed by a second oxidative burst more intense and sustained. On the other hand, a compatible interaction will only induce the first burst. Therefore, it seems that only the second oxidative burst is correlated to disease resistance (Lamb and Dixon, 1997).

Several enzymatic mechanisms have been related to ROS generation including NADPH oxidase, superoxide dismutase (SOD), oxalate oxidases, peroxidases, lipoxygenases and amine oxidases, as well as ROS producing organelles – mitochondria, chloroplasts and peroxissomes (Shetty *et al.*, 2008; Zhao *et al.*, 2005a). Although NADPH oxidase is the enzyme that received most attention in plant pathogen interactions, other enzymes were shown to produce ROS in response to pathogen in some plant systems (Hancock *et al.*, 2002).

In many cases it was shown to be necessary a $[Ca^{2+}]_{cyt}$ spike prior to ROS accumulation; in this way, NADPH oxidase have been shown to be directly activated by Ca^{2+} (Sagi and Fluhr, 2006), also its substrate NADPH may be produced by a Ca^{2+}/CaM-dependent NAD:NADH kinases (Hunt *et al.*, 2004).

ROS are thought to be crucial in plant defence, since they seem to be involved in quite a few defence processes. Although it is not fully understood the functions ROS play in these defence processes, several roles were suggested. Is has been proposed that ROS can be directly toxic to invading pathogens; particularly H_2O_2 has been considered to act as an antimicrobial agent (De Gara *et al.*, 2003; Shetty *et al.*, 2008). Also, ROS are involved in the strengthening of cell walls: the accumulation of H_2O_2, generated from oxidative burst at cell surface, induces rapid oxidative cross-linking of cell wall structural proteins, contributing to slow pathogen ingress and spread, and to trap pathogens in attacked cells that will probably undergo HR, thus enhancing the effectiveness of host cell suicide as a defence mechanism

(Brisson *et al.*, 1994). Moreover, ROS have been suggested to be involved in cell signalling at several levels and cross-talking with several pathways. ROS can activate Ca^{2+} channels leading to increased $[Ca^{2+}]_{cyt}$ (Mori and Schroder, 2004), that in its turn can lead to an enhancement of ROS production and in this manner amplify the defence response (Shetty *et al.*, 2008). Also, ROS seem to be involved in activating transcription of several defence-related genes, such as PR proteins and genes of the phenylpropanoid pathway leading to increased phytoalexin production. It is not clear how ROS mediate gene transcription, but it seems that H_2O_2 can activate transcription factors (TFs) by oxidizing its cysteine residues, or H_2O_2 can activate MAPKs that will phosphorilate and activate TFs (Desikan *et al.*, 2003; Shetty *et al.*, 2008). Furthermore, although not completely understood, ROS accumulation seems to be correlated with HR in many plant systems, but there are exceptions (Desikan *et al.*, 2003; Dorey *et al.*, 1999; Shetty *et al.*, 2008).

ROS have also been implicated in lipid peroxidation processes (Blokhina *et al.*, 2003), which can initiate the octadecanoid pathway generating oxylipins, jasmonic acid (JA) and other related compounds that have been implicated in defence gene expression leading to accumulation of plant secondary metabolites (Mehdy, 1994; Zhao *et al.*, 2005a). Besides cross talking with JA signalling pathways, ROS also seem to be involved in SA signalling. SA levels have been shown to increase in plants responding to pathogen attack, which might be the result of the activation of the phenylpropanoid pathway by ROS (Alvarez, 2000). On the other hand, SA has been implicated in the down-regulation of ROS scavenging systems, thus leading to increased ROS levels (Rao *et al.*, 1997; Shetty *et al.*, 2008). Additionally, ROS-SA crosstalk has been implicated in HR and SAR processes, since both SA accumulation and ROS bursts in infected tissues have been shown to be correlated with HR, and accumulation of SA and secondary oxidative bursts in uninfected parts of the plant might lead to microscopic HR lesions at these sites and seem to be required to SAR establishment (Alvarez, 2000; Dong, 1998; Durrant and Dong, 2004).

3.1.3 – Defence signalling

Activation of induced defence responses is costly for the plant. It has allocation costs (resources are used in defence instead of growth or reproduction), ecological costs (defence mechanisms expressed might interact negatively with plant ecological interactions, for example interactions with mutualistic organisms), and genetic costs (when resistance genes affect negatively fitness traits); plus, the costs are as much higher as limited are the resources available (Walters and Heil, 2007). The highly regulated signalling mechanisms used by plants, help them coordinating and mounting an appropriate defence response that can attain disease resistance with minimal costs (Koornneef and Pieterse, 2008).

The phytohormones SA and JA are considered key players in defence response regulation; although other hormones such as ethylene, abcisic acid, brassinosteroids and auxins are known to be involved, SA and JA are the best studied (Pieterse and Dicke, 2007).

There are some exceptions, but generally SA induced defences are more effective against virus and biotrophic pathogens (that need the host cell alive in order to feed or replicate) and JA mediated responses are more effective against necrotrophic pathogens and herbivores (Rojo *et al.*, 2003). However, in its natural habitat, the plants are exposed to several stresses simultaneously including different types of pathogens or herbivores and abiotic stressors, as well as symbiotic organisms that also elicit defence responses. Therefore, it is not surprising that several defence responses overlap and that no signalling molecule responds exclusively to only one stressor type: by extensive cross talking between signalling pathways, plants can achieve highly regulated defence responses against simultaneous aggressors and augment the velocity of the response while minimizing costs for the plant (Koornneef and Pieterse, 2008; Pieterse and Dicke, 2007).

The central role of SA in plant defence has been known for a long time: not only it was demonstrated that SA levels increase upon infection in attacked tissue and other parts of the plant, but also, application of SA or analogues enhances plant resistance by inducing SAR (Kunkel and Brooks, 2002;

Pieterse *et al.*, 2001). SA is known to be involved in HR by promoting rapid localized cell death at the site of infection; SA-induced defence is very effective against biotrophs. Also, SA is implicated in the establishment of SAR (that is thought to result from the concerted activation of a set of PR proteins), leading to the immunization of the entire plant against a large spectrum of pathogens (Alvarez, 2000; Dong, 1998; Kunkel and Brooks, 2002; Pieterse *et al.*, 2001).

JA and related compounds (for example., MeJ) have also been known to be transducers of elicitor signals for a long time, including playing roles in defence against biotic and abiotic stresses, in addition to signalization in plant development (Creelman and Mullet, 1997; Kunkel and Brooks, 2002). JA signalling pathways lead to the production of secondary metabolites, as well as to the induction of PR proteins (for instance, defensins or chitinases). Also, the external application of JA or related compounds leads to increased biosynthesis of secondary metabolites (Kunkel and Brooks, 2002; Zhao *et al.*, 2005a; Zhao and Sakai, 2003). Moreover, JA is capable of inducing systemic resistance (Heil and Bostock, 2002).

In the end, it is the interaction of SA and JA and other pathways that allow the plant to set up a successful defence response. Signalling pathways do not proceed in a linear way; instead, different pathways interact synergistically or antagonistically in order to respond appropriately to the challenges encountered, while trying to maintain the energy costs bearable (Koornneef and Pieterse, 2008; Pieterse and Dicke, 2007; Rojo *et al.*, 2003). Including, the crosstalk between pathways continues beyond successful response to a primary infection. Generally, SAR is induced by microbial pathogens through SA signalling, while herbivore attack leads to systemic resistance through JA signalling pathways. However, resistance induced by pathogens might be active against herbivores and *vice versa* (Heil and Bostock, 2002).

The complex and not fully understood information of signal transduction from stressor recognition to defence response in overviewed in Figure 3.2. Further clarification of these processes will help to achieve many goals, from

better products or practices for agricultural use, to better production processes of plant metabolites used in human health and nutrition.

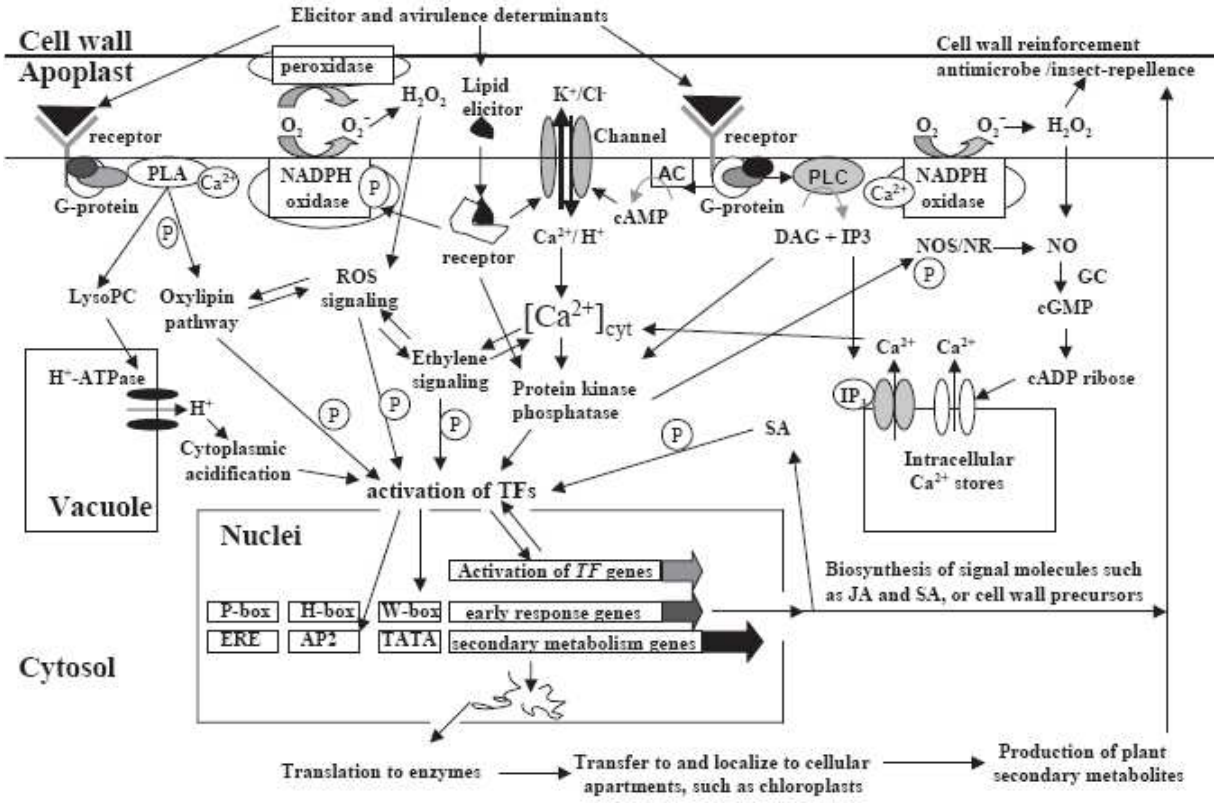

Figure 3.2 - *A comprehensive schematic illustration of elicitor signal transduction network. Proteinaceous or carbohydrate elicitor molecules are recognized by specific receptors on the plasma membrane. Guanosine triphosphate (GTP)-binding proteins (G-proteins) may be coupled to receptors and mediate elicitor-induced ion channel activation. Ion fluxes, especially Ca^{2+} influx, cause cytosolic free Ca^{2+} spiking which causes activation of protein kinases, peroxidases, NADPH oxidases, and phospholipases, which further generate other signaling messengers, such as reactive oxygen species, diacylglycerol (DAG), IP_3, cyclic adenosine monophosphate (cAMP), lysophosphatidylcholine (lysoPC), JA, ethylene, NO, cyclic adenosine diphosphate (cADP) ribose, and SA. All these messengers compose paralleling or crosslinking pathways to integrate these signals onto regulation of transcription factors. Various transcription factors integrate these signaling to activate gene expression by transcription machinery. Phenylpropanoid pathway is one of earlier induced pathways for cell wall reinforcement. Most genes for secondary metabolite synthesis are later response genes. The circled "P" shows protein phosphorylation and dephosphorylation-dependent regulation while the circled "Ca" shows Ca^{2+}-dependent regulation. Lipid elicitor such as syringolide or cerebroside could be perceived by receptors localized to the cytoplasm. Lipid elicitors also induce Ca^{2+} signaling and oxidative burst, and eventually stimulate the accumulation of plant secondary metabolites. NOS: nitric oxide synthase; NR: Nitrate reductase; AC: AMP cyclase; GC: guanosine monophosphate (GMP) cyclase. It is must be noted that a plant may only use parts of this elicitor signaling network to fulfill their responses to elicitors* (from Zhao *et al.*, 2005a).

3.2 - Results and Discussion

Calli cultures were established from leaf explants of *V. vinifera* cv. Vinhão. Leaf explants were used since esca symptoms *in vivo* are quite noticeable in foliar tissue and the cv. Vinhão was chosen because it is considered less susceptible to esca disease, probably being a better model to study eventual *V. vinifera* defence mechanisms against fungi associated with esca. The *calli* obtained were friable and easily dispersed in liquid medium (Figure 3.3). A homogeneous cells suspension culture composed by isolate or small clusters of 3-4 cells was established after several subcultures (5-7).

Figure 3.3 – *V. vinifera* cv. Vinhão cells suspension culture (left) and *calli* (right).

These suspension cultures proved to be stable, easy to manipulate, and showed an exponential growth phase between 3rd and 12th days after subculture. These cells suspensions were used in elicitation experiments.

Elicitation with *Pch* extract induced browning of liquid cultures after 3 days (Figure 3.4). However, cells remained viable during the entire period of the experiments and biomass production was similar to that of the control.

Figure 3.4 – *V. vinifera* cv. Vinhão cells suspension cultures control (left) and 72 hours after elicitation with *Pch* extract (right).

In spite of the well documented presence of esca disease in the field particularly since the 1980's (and even before), the studies on *Vitis* defence mechanisms to esca are still scarce. A main reason for the lack of information is the inherent complexity of the disease, in which several fungi seem to be involved and the symptoms presented are inconstant from year to year due to the high influence of environmental factors (Surico *et al.*, 2006). In this scenario, host-pathogens studies in the field are very difficult. Field studies may take several years of observations (Bruno and Sparapano, 2006b; Marchi *et al.*, 2006; Sparapano *et al.*, 2001a). The disadvantages of host-pathogens field studies were previously discussed in the literature

(Smalley and Guries, 1993): results may be misleading due to seasonal influences, results do not separate pathogen effects from effects induced by other biotic and/or abiotic factors present, and field experiments of woody plants are often time consuming. According to these authors, it would be more appropriate to the study of host-pathogen interactions the use of short term assays in controlled conditions, including the combination of *in vitro* cultures with conventional ones; also, the use of *in vitro* cultures allows large screenings in short periods of time and occupation of small areas. So, *in vitro* cultures could represent an opportunity to study the defence mechanisms against esca, given its advantages that include the simplicity of manipulation and the better control of external factors that can interfere with the whole-plant tissue metabolic responses, enabling a precise cause-effect determination. *In vitro* cultures of plants have been utilized successfully as models to study biochemical changes related to plant defence against pathogens (Hahlbrock *et al.*, 2003), including to study interactions between grapevine and esca-related fungi and that were shown to result in decrease of *calli* growth, appearance of both symptoms and host response in a few weeks, and the distinction of susceptible and less susceptible cultivars (Bruno and Sparapano, 2006a; Santos *et al.*, 2005; Sparapano *et al.*, 2001b; Zanzotto *et al.*, 2008). However, these *in vitro* studies of esca disease, particularly concerning grapevine defence mechanisms, are scarce. Nonetheless, the few *in vitro* experiments published are consistent with *ex vitro* results, suggesting that *in vitro* assays may replace, at least as a first approach, the field studies (Santos *et al.*, 2005 and references therein).

3.2.1 – Modulation of phenolic production in *V. vinifera* cv. Vinhão cell cultures upon elicitation with *Pch*, SA and MeJ

The modulation of phenolic production in *V. vinifera* cv. Vinhão cells suspensions induced by *Pch* elicitation was analysed, as well as the effect of SA and MeJ elicitation and priming. HPLC profiles of *V. vinifera* cv. Vinhão

cells methanolic extracts, at 306nm, are shown in Figure 3.5. The figure shows representative HPLC profiles of grapevine cells elicited with SA, MeJ and *Pch*, as well as *Pch* elicited cells primed with SA or MeJ 24 hours before.

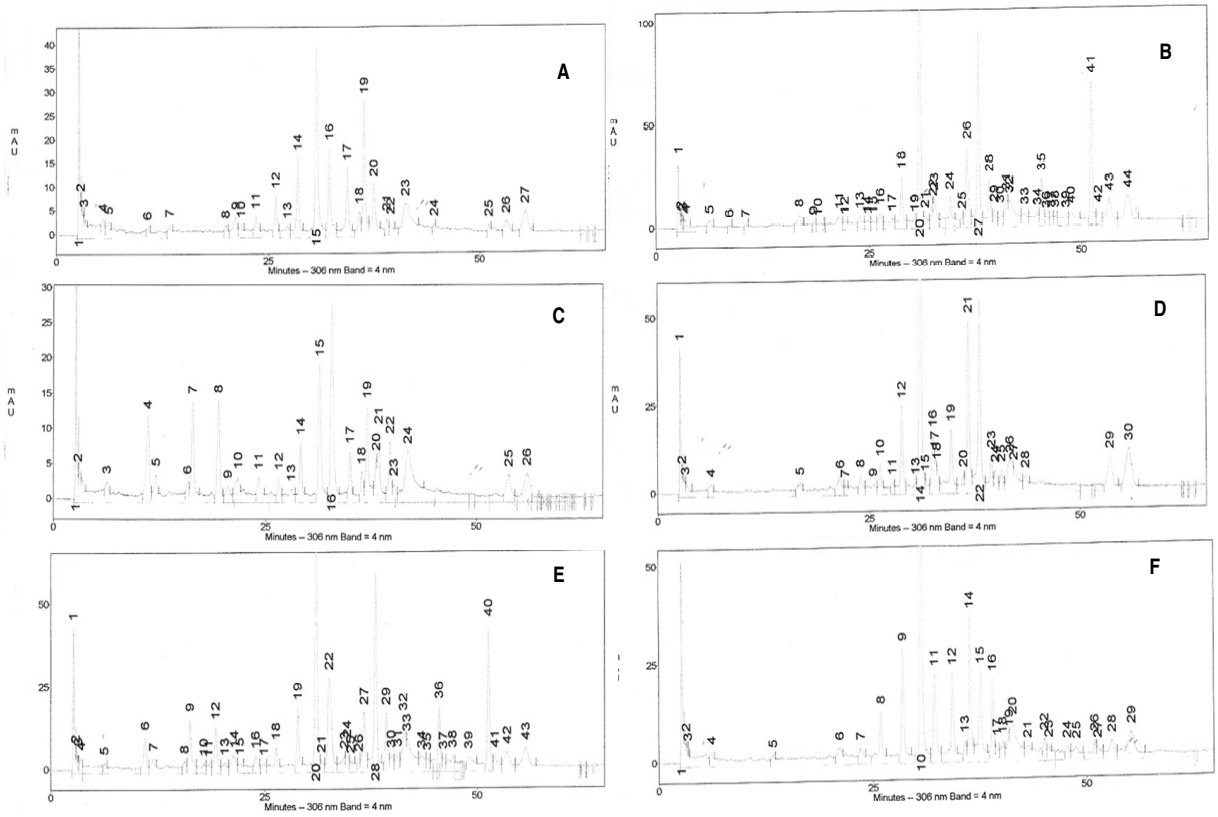

Figure 3.5 - HPLC profiles of *V. vinifera* cv. Vinhão cell cultures methanolic extracts, 306nm, 24 hours after fungal elicitation. A – Control; B – MeJ (100µM); C – SA (100µM); D – MeJ (100µM) + *Pch* extract (0.5mg/ml); E – SA (100µM) + *Pch* extract (0.5mg/ml); F – *Pch* extract (0.5mg/ml).

Total stilbenic production was quantified at 24 and 72 hours after fungal elicitation (Figure 3.6).

When compared to control, results show a significant increase in stilbenic production of *V. vinifera* cv. Vinhão cell cultures in a short period (24 hours) after elicitation with MeJ, *Pch* and priming with MeJ before fungal elicitation. Priming with SA also leads to increased stilbenic production when compared to control, but lower when compared to *Pch* elicitation alone. Stilbenic

production decreases 72 hours post fungal elicitation but remains significantly higher than control in cultures elicited MeJ, *Pch* and primed with MeJ before fungal elicitation. Elicitation or priming with MeJ always induces the highest stilbenic production levels, including higher levels than elicitation with *Pch* alone.

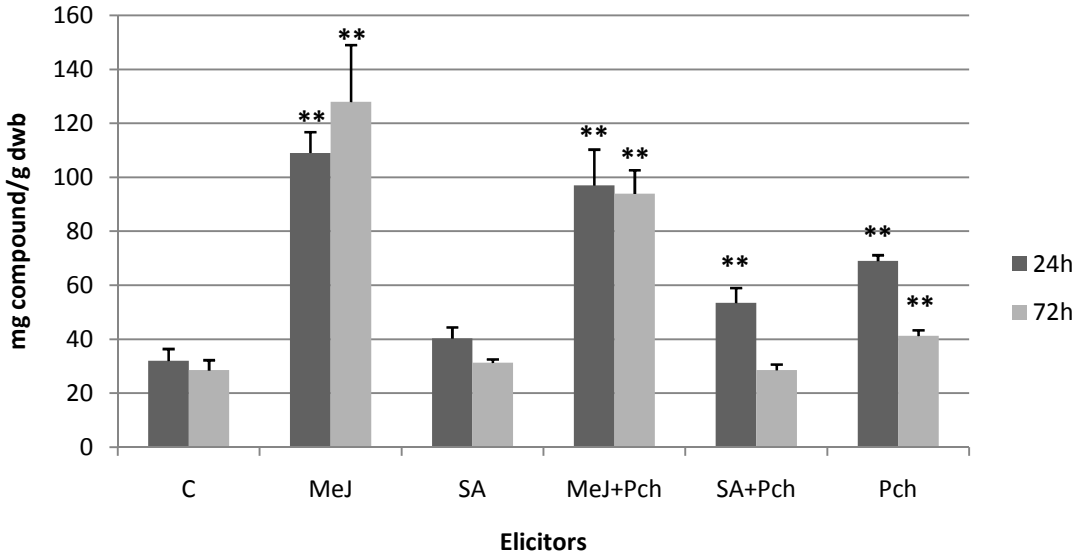

Figure 3.6 – Total stilbenic production of *V. vinifera* cv. Vinhão cell cultures at 24 and 72 hours after *Pch* elicitation. C – Control; MeJ – MeJ (100µM); SA – SA (100µM); MeJ+*Pch* – MeJ (100µM) + *Pch* extract (0.5mg/ml); SA+*Pch* – SA (100µM) + *Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three independent replicates (experiment was repeated twice). Asterisks indicate significant statistical differences relative to control (**p<0.01).

Because HPLC profiles of *V. vinifera* cv. Vinhão cells methanolic extracts at 520nm (Figure 3.7) also seemed different between the treatments tested, anthocyanin content was also evaluated. The Figure 3.7 shows representative HPLC profiles of cells elicited with SA, MeJ and *Pch*, as well as *Pch* elicited cells primed with SA or MeJ 24 hours before. Total anthocyanin production quantified at 24 and 72 hours after fungal elicitation is shown in Figure 3.8.

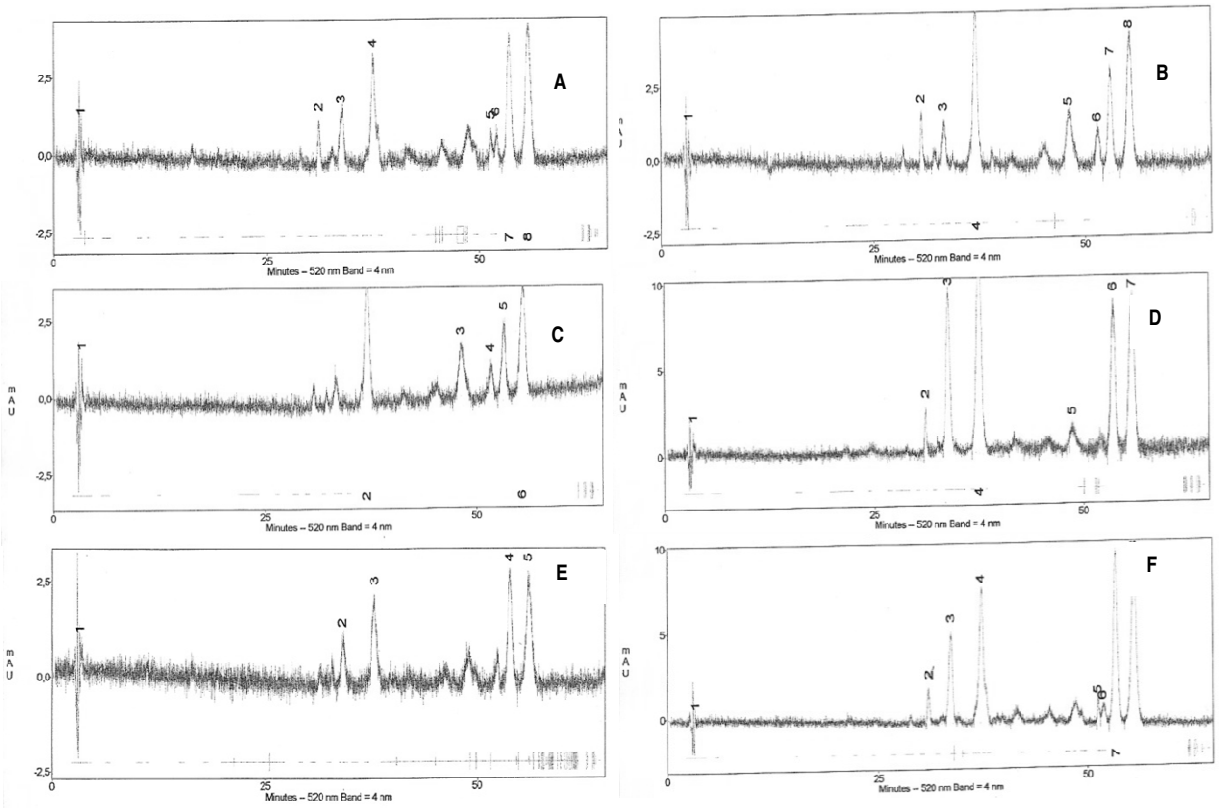

Figure 3.7 - HPLC profiles of *V. vinifera* cv. Vinhão cell cultures methanolic extracts, 520nm, 24 hours after fungal elicitation. A – Control; B – MeJ (100µM); C – SA (100µM); D – MeJ (100µM) + *Pch* extract (0.5mg/ml); E – SA (100µM) + *Pch* extract (0.5mg/ml); F – *Pch* extract (0.5mg/ml).

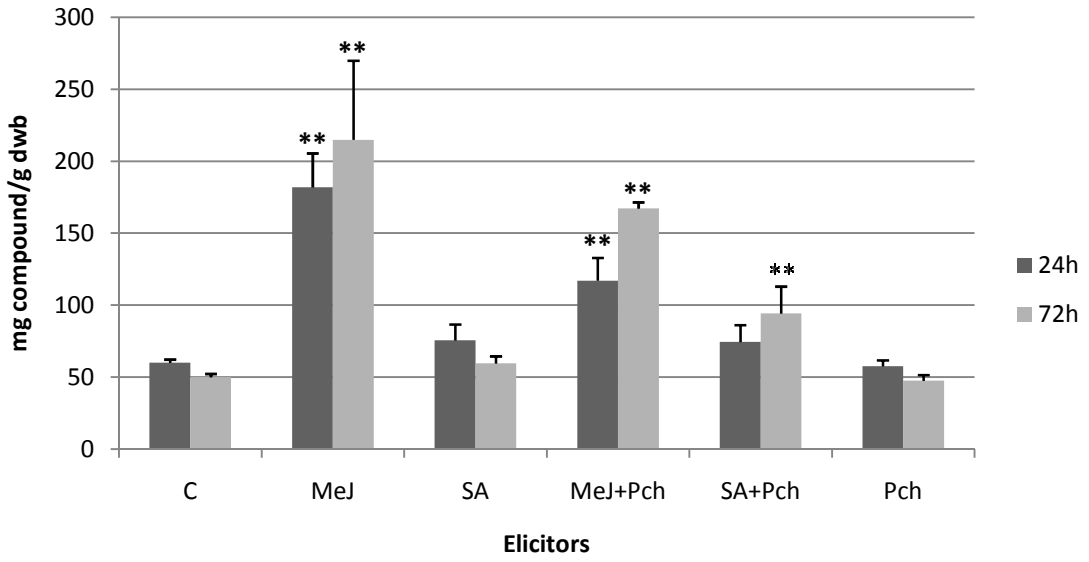

Figure 3.8 - Total anthocyanin production of *V. vinifera* cv. Vinhão cell cultures, 24 and 48 hours after *Pch* elicitation. C – Control; MeJ – methyl jasmonate (100µM); SA – salicylic acid (100µM); MeJ+*Pch* – methyl jasmonate (100µM) + *P. chlamydospora* extract (0.5mg/ml); SA+*Pch* – salicylic acid (100µM) + *P. chlamydospora* extract (0.5mg/ml); *Pch* – *P. chlamydospora* extract (0.5mg/ml). Results are mean ± standard deviation of three independent replicates (experiment was repeated twice). Asterisks indicate significant statistical differences relative to control (**p<0.01).

The results indicate that anthocyanin levels do not increase upon fungal elicitation alone. However, anthocyanins increase in cells elicited or primed with MeJ. Priming with SA also increases anthocyanin levels, but only 72 hours after fungal elicitation. In the treatments that induce anthocyanins increase, anthocyanins levels seem to be sustained and seem to increase overtime. Again, MeJ appears to have an essential role in the process.

SA and JA have been shown to accumulate upon infection or elicitation and to be involved in signal cascades leading to the production of various defence mechanisms (e.g. phytoalexin production), as well as to the induction of systemic resistance (Alami *et al.*, 1999; van Loon *et al.*, 1998). It has been proposed to name Systemic Acquired Resistance (SAR) the systemic resistance mediated by SA, and Induced Systemic Resistance (ISR) to refer to systemic resistance dependent of JA signalling pathways (Goellner and Conrath, 2008; van Loon *et al.*, 1998).

Systemic resistance acquired in a primary infection allows plants to enhance the velocity and/or intensity of defence responses to a second challenge posed by a broad range of biotic or abiotic stressors (Goellner and Conrath, 2008). The plant is said to be in a *primed state*, which means: *a physiological condition in which plants are able to better or more rapidly mount defence responses, or both, to biotic or abiotic stresses* (Conrath *et al.*, 2006).

Induced resistance is a process that occurs naturally in response to virulent pathogens, but also can be elicited by avirulent or non-pathogens (van Loon *et al.*, 1998). SA and JA (and related compounds such as MeJ) have been used as priming agents in several plant models, ranging from cells suspension cultures to leaves and other plant tissues, and have been shown to potentiate defence responses to subsequent elicitation, including enhancement of ROS production, PAL synthesis and increased phytoalexin

production (Belhadj *et al.*, 2006; Clérivet and Alami, 1999; Conceição *et al.*, 2006; Conrath *et al.*, 2002; Gundlach *et al.*, 1992; Shirazu *et al.*, 1997).

The results show that MeJ priming potentiates phenolic production. It is not surprising that MeJ and not SA induces the highest phenolic production since JA signalling pathways have been consistently linked to increase of secondary metabolites production (Alami *et al.*, 1999; Clérivet and Alami, 1999; Gundlach *et al.*, 1992), including the increase of stilbenes in grapevine cells suspensions (Belhadj *et al.*, 2008; Tassoni *et al.*, 2005), probably because jasmonates have been shown to induce expression of phenylpropanoid pathway genes (Belhadj *et al.*, 2006; Belhadj *et al.*, 2008; Ellard-Ivey and Douglas, 1996). For example, elicitation of *Rauvolfia* and *Eschscholtzia* suspension cultures with a fungal elicitor results in rapid synthesis of jasmonate and its methyl ester; additionally, exogenous application of MeJ induces the synthesis of phytoalexins in several plant species, as well as induces *de novo* transcription of genes, including PAL (the key enzyme in the phenylpropanoid pathway) (Gundlach *et al.*, 1992). Application of MeJ to grapevine leaves results in lesions that mimic typical HR, and in the activation of associated defence responses such as increased production of salicylic acid, accumulation of phenolic compounds and expression of defence-related genes (Repka *et al.*, 2001). Moreover, elicitation of *V. vinifera* leaves, grapes or cells suspension cultures with MeJ results in phytoalexin accumulation, namely stilbenes including resveratrol and ε-viniferin (Belhadj *et al.*, 2006; Belhadj *et al.*, 2008; Krisa *et al.*, 1999; Tassoni *et al.*, 2005; Vezzulli *et al.*, 2007).

The quantification of anthocyanins confirms the capacity of MeJ to enhance phenolic production, and doing so in various phenolic groups: not only increasing stilbenic compounds, but also anthocyanins. The capacity of jasmonates to increase anthocyanin production in *V. vinifera* cells was already documented (Belhadj *et al.*, 2008; Curtin *et al.*, 2003; Zhang *et al.*, 2002). Although traditionally the roles attributed to anthocyanins are restricted to their function in reproduction (as colourful attractants of pollinators or seed dispersal agents) or in abiotic stress (as protectors of plant cells against strong light or extreme temperatures, for example), it has

been recently proposed that anthocyanins may take part in defence against biotic stress (Hatier and Gould, 2008). Anthocyanins are proposed to modulate defence signal cascades by directly scavenging generated ROS and other free radicals, by protecting cellular components of free radicals or/and by interacting with other molecules involved in defence response (Hatier and Gould, 2008). So, despite anthocyanin levels remain at control levels after fungal elicitation, a defensive role of anthocyanins cannot be ruled out.

The results seem to indicate that MeJ priming might be an efficient strategy to protect grapevine, at least in *V. vinifera – Pch* interaction. The protection of crops has always been a concern in agriculture and led to extensive and intensive use of chemical fungicides. Nowadays, environmental, health and food safety concerns are leading to the search of alternative protection strategies (Belhadj *et al.*, 2008). In this way, the induction of plants' natural defences by inducing systemic resistance, such as SAR or ISR, have been found attractive due to their broad spectrum of activity against virus, bacteria and fungi, and this has been done by applying SA, JA or chemical analogues to crops (Vallad and Goodman, 2004; Walters and Heil, 2007). Since the activation of a full set of defences is costly for the plants, priming is considered a more advantageous strategy: primed plants stay in an alert state that allows them to promptly mobilize defences, but are not constitutively expressing defences and therefore the costs on growth and reproduction are minimized (Vallad and Goodman, 2004; Walters and Heil, 2007). MeJ has already been used with positive results in pest management (Yao and Tian, 2005), including MeJ potential has already been proposed in grapevine protection (Belhadj *et al.*, 2006; Belhadj *et al.*, 2008). However, induced resistance is not satisfactory to use as only method but in an integrated pest management that includes also chemical pesticides. Nonetheless, it encloses the potential of reducing the use chemical pesticides leading to environmental, health and safety advantages. Still, much work has to be done to define the proper doses and moments of application of systemic resistance elicitors (Vallad and Goodman, 2004).

3.2.2 – Changes in phenolic production of *V. vinifera* cv. Vinhão cell cultures induced by *Pch* and MeJ

Differential changes in phenolic production induced by *Pch* and MeJ in *V. vinifera* cv. Vinhão cell cultures was analysed. HPLC profiles of *V. vinifera* cv. Vinhão cells methanolic extracts, at 306nm, are shown in Figure 3.9. The figure shows representative HPLC profiles of grapevine cells after elicitation with MeJ and *Pch*, and several stilbenic compounds are identified.

In control cells cultures the main compounds produced are the piceids: *trans*-piceid-glucoside, *trans*-piceid, 3-hydroxy-methoxy-stilben-glucoside and *cis*-piceid, identified in Figure 3.9.

Both elicitations with *Pch* and MeJ induce significant changes in phenolic production, when compared to control profiles. Along with the piceid compounds *trans*-piceid-glucoside, *trans*-piceid and *cis*-piceid, elicitation with *Pch* also induces *de novo* production of viniferin compounds, identified by HPLC-MS as a possible viniferin, ε-viniferin-2-glucoside, ε-viniferin-glucoside and a two molecule ε-viniferin polymer (Figure 3.9-B).

MeJ elicitation also induces *de novo* production of ε-viniferin-2-glucoside, ε-viniferin-glucoside and a two molecule ε-viniferin polymer, along with the piceids *trans*-piceid-glucoside, *trans*-piceid and *cis*-piceid (Figure 3.9-C).

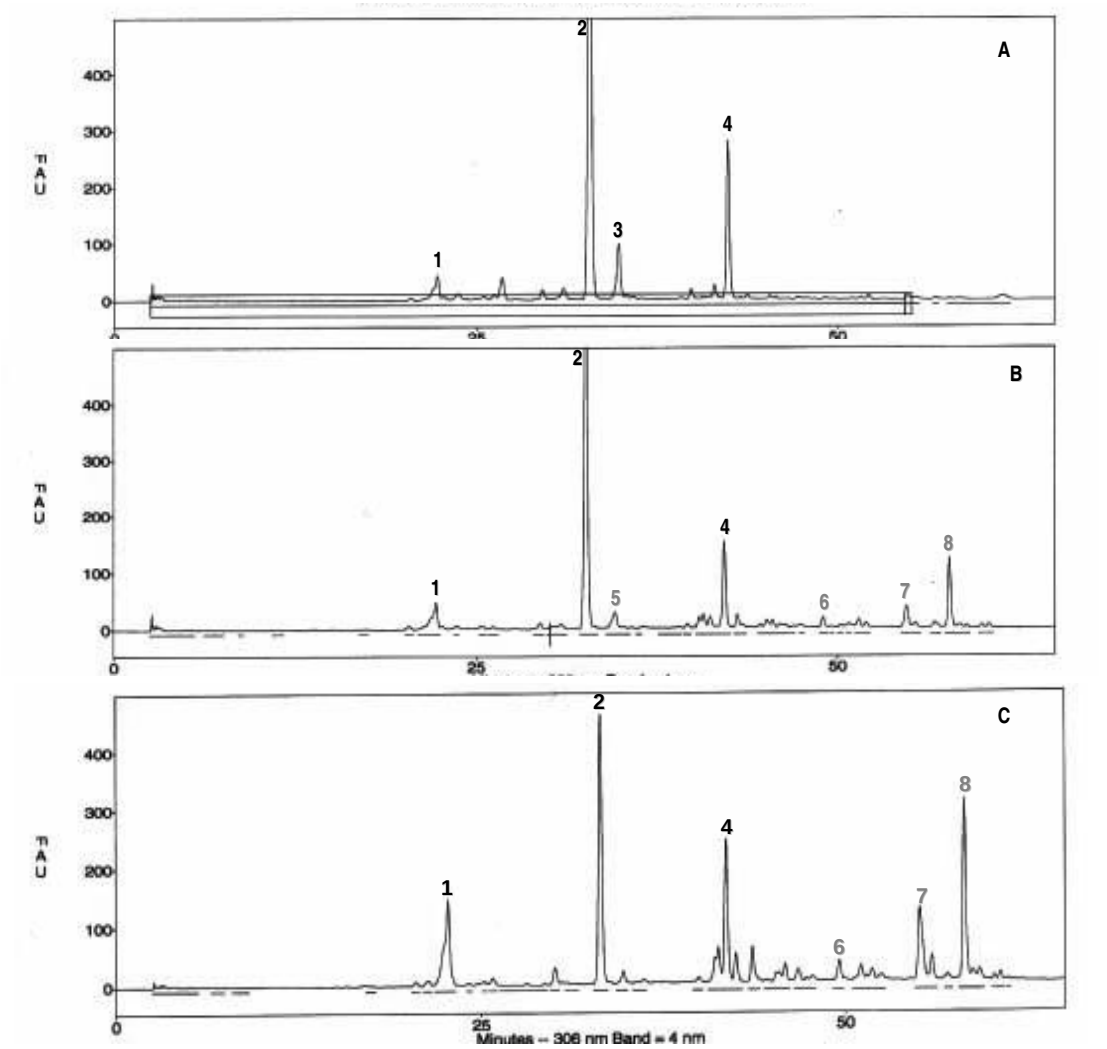

Figure 3.9 - HPLC profiles of *V. vinifera* cv. Vinhão cell cultures methanolic extracts, 306nm, 72 hours after elicitation. A – Control; B – *Pch* extract (0.5 mg/ml); C – MeJ (100 μM). 1 - *trans*-piceid-glucoside; 2 - *trans*-piceid; 3 – 3-hydroxy-methoxy-stilben-glucoside; 4 - *cis*-piceid; 5 - viniferin (?); 6 – ε-viniferin-2-glucoside; 7 – ε-viniferin-glucoside; 8 – ε-viniferin polymer (2 molecules). Black numbers: piceid type compounds; grey numbers: viniferin type compounds.

Total phenolic quantification of identified compounds, shows a significant increase when elicitation occurs (Figure 3.10). MeJ elicitation induces a 9-fold increase of identified compounds while *Pch* induces a more marked increase of 20-fold in these compounds, comparatively to control conditions. Total piceid production increases from 31.88 μg/g dry weight biomass (dwb) in control cell cultures to 183.90 μg/g dwb in MeJ elicited cells and 456.24 μg/g dwb in cells elicited with *Pch*. Also, *de novo* viniferin production occurs

at a noteworthy level when cell cultures are elicited with MeJ (110.00 µg/g dwb) or elicited with fungal extract (188.55 µg/g dwb); the total viniferin production in these two conditions is not significantly different from each other.

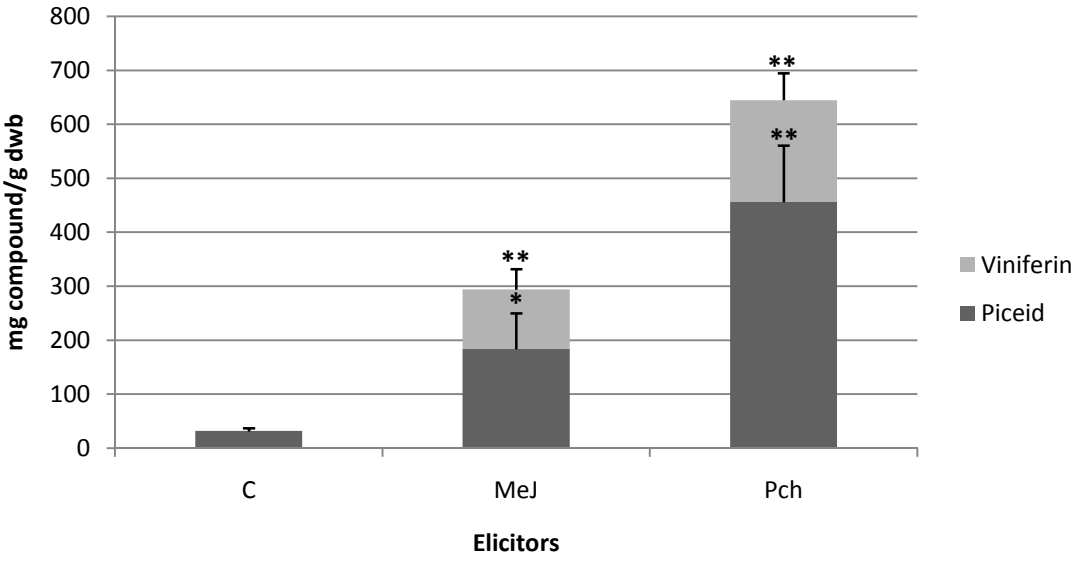

Figure 3.10 - Total phenolic production of *V. vinifera* cv. Vinhão cell cultures. C – Control; MeJ – MeJ (100µM); *Pch* – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three independent replicates (experiment was repeated twice). Asterisks indicate significant statistical differences relative to control (* $p<0.05$; ** $p<0.01$).

The results show that elicitation of *V. Vinifera* cv. Vinhão cell cultures with *Pch* and MeJ induces changes in phenolic production, including *de novo* production of three viniferin type compounds identified as ε-viniferin-2-glucoside, ε-viniferin-glucoside and a polymer of two ε-viniferin molecules. The synthesis of phenolic phytoalexins is a recognized defence mechanism in plants, namely in grapevine. The contribution of phenylpropanoid compounds to disease limitation has been shown: the insertion of grapevine stilbenes synthase gene in several plants (tobacco, rice, tomato, alfafa, kiwifruits, barley wheat and grapevine) led to an increased production of stilbenic phytoalexins and/or increased resistance to several pathogens;

conversely, the suppression of the first enzyme of the phenylpropanoid pathway (PAL) in tobacco plants led to higher susceptibility to fungal infection (Jeandet *et al.*, 2002; Maher *et al.*, 1994). In *Vitis* spp. phenolic substances accumulate in response to abiotic or biotic elicitors such as UV irradiation (Adrian *et al.*, 2000; Douillet-Breuil *et al.*, 1999; Langcake and Pryce, 1977), or bacterial and fungal pathogens such as *Pseudomonas syringae* (Robert et al., 2001), the causal agent of grey mould *Botrytis cinerea* (Goetz *et al.*, 1999; Jeandet *et al.*, 1995), the berry rot *Rhizopus stolonifer* (Sarig *et al.*, 1997) and the downy mildew *Plasmopara viticola* (Dai *et al.*, 1995a). In grapevine, resveratrol and related compounds (viniferins) are the major forms of phytoalexins (Jeandet *et al.*, 2002). Particularly, the inoculation of *V. vinifera* with *P. syringae, B. cinerea* or *P. viticola* led to an increase in resveratrol production (Dai *et al.*, 1995a; Jeandet *et al.*, 1995; Robert *et al.*, 2001). Additionally, *B. cinerea* stilbene oxidase (enzyme important in *B. cinerea* pathogenesis against grapevine) was shown to be inhibited by the constitutive berry phenolic compounds: catechin, epicatechin-3-*O*-gallate, taxifoline-3-*O*-rhamnoside, quercetin-3-*O*-glucuronide and *trans*-caftaric, *trans*-coumaric and *trans*- and *cis*-coutaric acids (Goetz *et al.*, 1999). Also, resveratrol was shown to have antifungal activity against *B. cinerea in vitro* (Adrian *et al.*, 1997) and exogenous application of resveratrol to grapes was shown to maintain post-harvest quality and increase normal shelf-life (Montero *et al.*, 2003). Inoculation of grape berries with *R. stolonifer* also led to the accumulation of resveratrol and pterostilbene (Sarig *et al.*, 1997). Recently, changes in phenolic production of grapevine *calli* in response to elicitation with *Pch* have been reported (Bruno and Sparapano, 2006a). Also, grape roots infected with *Pch*, showed increased polyphenolic content, including increased resveratrol, and extracts of these infected roots were shown to inhibit *Pch* and *Phaeoacremonium* spp. mycelia growth (Del Rio *et al.*, 2001). In both studies, the higher capacity to produce phenolic compounds seemed to be related to lower disease susceptibility.

Therefore, it is possible that the viniferins produced *de novo* by *V. vinifera* cv. Vinhão cells might be involved in the defence mechanism against *Pch*,

since they have been described as antifungal compounds; essentially, viniferins have been shown to be more fungitoxic than resveratrol and, in this way, resveratrol is thought to function as a precursor of viniferins (Jeandet *et al.*, 2002). Specifically, it was described an increase in the *in vivo* production of *trans*-resveratrol and ε-viniferin in the wood of grapevines showing esca symptoms (Amalfitano *et al.*, 2000).

The importance of MeJ in grapevine defence was already discussed in the previous section (3.2.1), and those results already indicated that MeJ may play an important part in *V. vinifera*-*Pch* interaction. Here, MeJ induced the production of the same phenolic compounds stimulated *de novo* by *Pch* elicitation. Again, the results indicate that MeJ may be directly involved in the defence of grapevine against *Pch*. In face of these results, further studies should be conducted to evaluate the potential of MeJ as a protecting agent in vines where esca disease is present.

3.2.3 – Oxidative response of *V. vinifera* cv. Vinhão cell cultures induced by *Pch*: influence of SA and MeJ

The time course ROS accumulation in *V. vinifera* cv. Vinhão cells cultures after elicitation with *Pch* extract was monitored and is shown in Figure 3.11. The influence of priming with SA and MeJ in the pathogen-induced oxidative burst was also analysed (Figure 3.11).

The ROS levels in *V. vinifera* cv. Vinhão cells cultures were detected through H_2DCF-DA probe. The use of H_2DCF-DA assay to access ROS levels relies in the theory that this non-polar, non-anionic probe crosses the cell membranes entering the cells, where intracellular esterases hydrolyse it to the non-fluorescent compound H_2DCF. This is rapidly oxidized in the presence of ROS to DCF, which is highly fluorescent – the fluorescence emitted is proportional to ROS levels present (LeBel *et al.*, 1992). The results of ROS accumulation are shown as the percentage of fluorescence

increase relative to the time of *Pch* elicitation (0 minutes), calculated as described in Chapter 2 – Material and Methods. Analysing percentage of fluorescence increase instead of net changes in fluorescence has advantages: the calculated data reflects directly the percentage changes in fluorescence overtime and cancels the background fluorescence, making unnecessary the use of "no cell" control (Wang and Joseph, 1999).

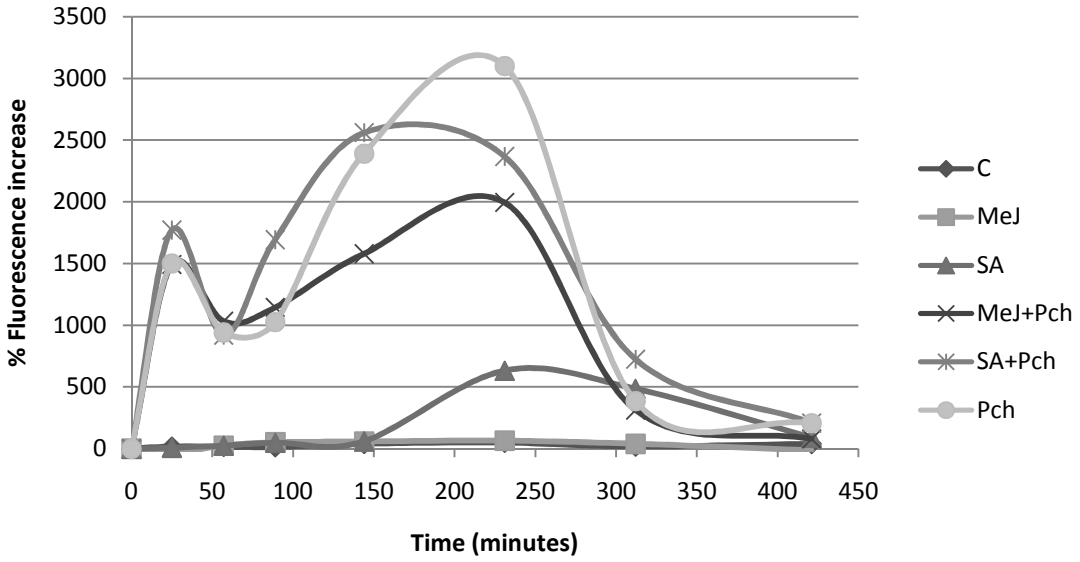

Figure 3.11 – ROS levels indicated by fluorescence increase (%) relative to 0 minutes (corresponding to fungal elicitation) in *V. vinifera* cv. Vinhão cell cultures. C – Control; MeJ – MeJ (100µM); SA – SA (100µM); MeJ+*Pch* – MeJ (100µM) + *Pch* extract (0.5mg/ml); SA+*Pch* – SA (100µM) + *Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results represent typical data obtained calculating % of fluorescence increase from the average of duplicates (experiment was repeated three times).

The results show the presence of a biphasic oxidative burst every time *Pch* extract is added to the cell cultures. A first rapid and smaller burst peaks around 20 minutes after fungal elicitation and is followed by a second longer and intense burst that shows a maximum around 230 minutes (sooner in the case of cells primed with SA 24 hours before *Pch* elicitation). Although ROS accumulation seems to be potentiated in the first phase of the burst by SA

priming, the priming with both SA and MeJ seems to be obstructive for ROS accumulation by the time of second phase of the oxidative burst.

The accumulation of ROS in cell cultures elicited with MeJ alone is similar to control, while ROS accumulation in cell cultures elicited with SA alone is similar to control until around 150 minutes after elicitation, but some ROS accumulation is detected after this time.

In plants, ROS occur as unavoidable by-products of normal metabolic reactions. As such, plants are equipped with an array of antioxidant or detoxifying enzymes that effectively control intracellular ROS levels. However, in stressful conditions, the protective mechanisms are overridden and cell components are activated leading to a rapid, intense and transient increase of ROS levels – the oxidative burst (Wojtaszek, 1997)

The presence of an oxidative burst at the onset of an infection was described for the first time when potato tuber tissue was inoculated with an incompatible race of *Phytophthora infestans* (Doke, 1983*)*. Today, the rapid generation of ROS is considered one of the earliest events following elicitation (Lamb and Dixon, 1997). Nonetheless, the information published in the literature concerning pathogen-induced oxidative burst is very diverse, concerning time of occurrence and intensity, depending on plant system studied or challenging factor used; usually, ROS accumulation appears sooner in cell cultures (within a few minutes) than in whole tissues or *in vivo* conditions (in which oxidative burst occurs after several hours) (Wojtaszek, 1997). Generally, compatible interactions induce a single nonspecific burst while incompatible interactions provoke a biphasic oxidative burst, the second phase of the burst being correlated to disease resistance (Lamb and Dixon, 1997); however, three-phase ROS accumulation have been observed (Shetty *et al.*, 2008). For example, when an aggressive and a non-aggressive *B. cinerea* isolate was used to inoculate French bean cell suspensions, both induced an early oxidative burst, but a second stronger burst was only present when cells were inoculated with the non-aggressive isolate, that resulted in an incompatible interaction (Unger *et al.*, 2005). It is believed that the first unspecific burst originates in pre-existing systems of

the plant (therefore being present after challenge with either compatible or incompatible elicitors), and that the second burst is a result of successfully activated defence response (Unger *et al.*, 2005).

Here, elicitation of grapevine cells with *Pch* extract leads to a biphasic-shaped oxidative burst. It should be noted that *V. vinifera* cv. Vinhão is a grapevine variety considered tolerant to esca disease. The biphasic ROS production has been correlated with disease resistance and was reported in several incompatible interactions, for instance, in tobacco leaves infected with tobacco mosaic virus (TMV) (Barna *et al.*, 2003), in tobacco cells suspensions inoculated with *Phytophthora nicotianae* (Able *et al.*, 2000), and in the above mentioned incompatible interaction of bean cells cultures and *B. cinerea* (Unger *et al.*, 2005).

Published information on MeJ elicitation and priming has been reaching different conclusions in different plant systems. Here, in *V. vinifera* cv. Vinhão cell cultures MeJ elicitation does not induce ROS accumulation, contrarily to a report in which MeJ induced ROS production within a few hours after elicitation (Zhang and Xing, 2008). Also, 24 hour MeJ pre-treatment was shown to increase fungal elicitors-induced ROS production in parsley cells suspensions (Kauss *et al.*, 1994). Here MeJ priming does not seem to interfere with the first phase of the burst of *Pch*-induced ROS accumulation and decreases the intensity of the second phase of the induced burst. This might happen due to the presence of antioxidant or ROS scavenging compounds induced by MeJ. It was already shown in the previous sections 3.2.1 and 3.2.2 that, in this experimental system, priming with MeJ strongly enhances secondary metabolite production, including an increase in anthocyanins (which antioxidant capacity is known). Also, it has been shown that MeJ treatment induced *de novo* biosynthesis of vitamin C in *Arabidopsis* and tobacco cell suspensions and it is known that vitamin C can directly scavenge ROS or serve as a substrate for ROS scavenging enzymes (Wolucka *et al.*, 2005).

SA has been thought to enhance oxidative burst since it was reported to specifically inhibit catalase activity and induced H_2O_2 increase (Chen *et al.*,

1993). However, it has also been described the incapability of SA to inhibit catalase (Summermatter *et al.*, 1995) and, therefore, SA may be acting through a different mechanism to elicit ROS increase. Although an immediate increase in ROS after addition of SA has been reported in tobacco cells suspensions (Kawano and Muto, 2000), in the results presented here SA only induces ROS accumulation higher then control after 150 minutes post elicitation. On the other hand, SA priming seems to potentiate the first oxidative burst induced by *Pch* (20 minutes after elicitation). Enhancement of ROS accumulation at 30 minutes after addition of fungal elicitors to parsley suspension cultures or hypocotyls of cucumber previously treated with SA has been reported (Fauth *et al.*, 1996; Kauss and Jeblick, 1995). However, SA priming seems to interfere with *Pch*-induced ROS accumulation in the second phase of the burst. The attenuation of biphasic ROS accumulation was already detected in TMV-infected tobacco leaves, previously treated with SA (Barna *et al.*, 2003).

3.2.4 - Possible involvement of Catalase, Peroxidase and NADPH oxidase in the oxidative burst

Several enzymes have been implicated in the pathogen-induced oxidative burst. Catalase is capable of regulating ROS levels in the cell by catalyzing the conversion of H_2O_2 to O_2 and water. So, catalase activity was measured as the capacity of cells to degrade H_2O_2 and calculated as O_2 appearance rate. Because the objective was to analyse if catalase contributed to the detoxification of ROS produced in the first and second phases of ROS accumulation, catalase activity was investigated at 20 and 230 minutes after *Pch* elicitation – the times at which ROS levels start falling. The results of catalase activity 20 and 230 after *Pch* extract elicitation are shown in Figure 3.12.

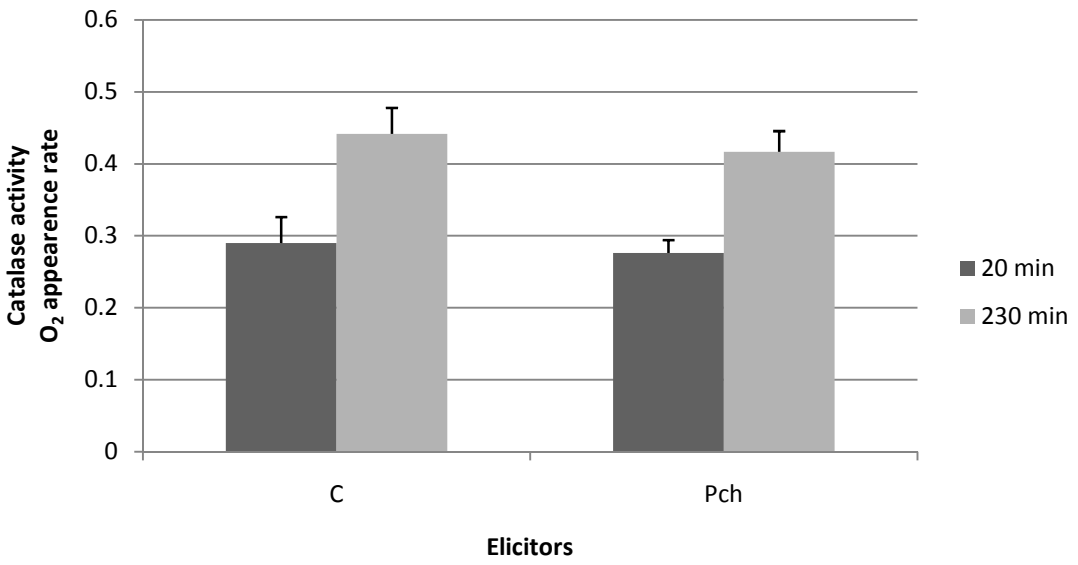

Figure 3.12 – Catalase activity measured as O2 appearance rate, at 20 and 230 minutes after fungal elicitation of V. vinifera cv. Vinhão cell cultures. C – Control; Pch – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three replicates (experiment was repeated twice).

Results show no significant difference in catalase activity between control and *Pch* elicited cultures. This may indicate that in *Pch-V. Vinifera* cv. Vinhão interaction other type of detoxifying systems must be regulating ROS production.

An absence in modification of catalase activity upon infection was already demonstrataed in other plant systems. Catalase activity was shown not to be altered in *Arabidopsis thaliana* leaves inoculated with *P. syringae* (Summermatter *et al.*, 1995), in tobacco leaves infected with TMV (Fodor *et al.*, 1997), in wheat sheat tissue after *Fusarium* infection (Kwon and Anderson, 2001), and in barley leaves in response to powdery mildew (Harrach *et al.*, 2008). However, other systems have been described in which catalase seems to play a role in defence. For example, it was shown that catalase activity increases in several maize tissues upon infection with *Aspergillus flavus* and that the increase in catalase activity is correlated to disease resistance (Magbanua *et al.*, 2007); on the other hand, a decline in catalase activity was described in *Pinus pinaster* cells elicited with the non-host pathogen *B. cinerea* (Azevedo et al., 2008).

For the generation of ROS during stress-induced oxidative burst, two major mechanisms have been proposed: cell wall peroxidase or plasma membrane NADPH oxidase (Bolwell *et al.*, 2002; Bolwell *et al.*, 1998). Plants can use either one of these mechanisms, or both (Bindschedler *et al.*, 2006).

To investigate if ROS in *Pch*-induced oxidative burst derived from peroxidase, peroxidase activity was measured at 30, 90, 180 and 300 minutes after *Pch* elicitation (Figure 3.13).

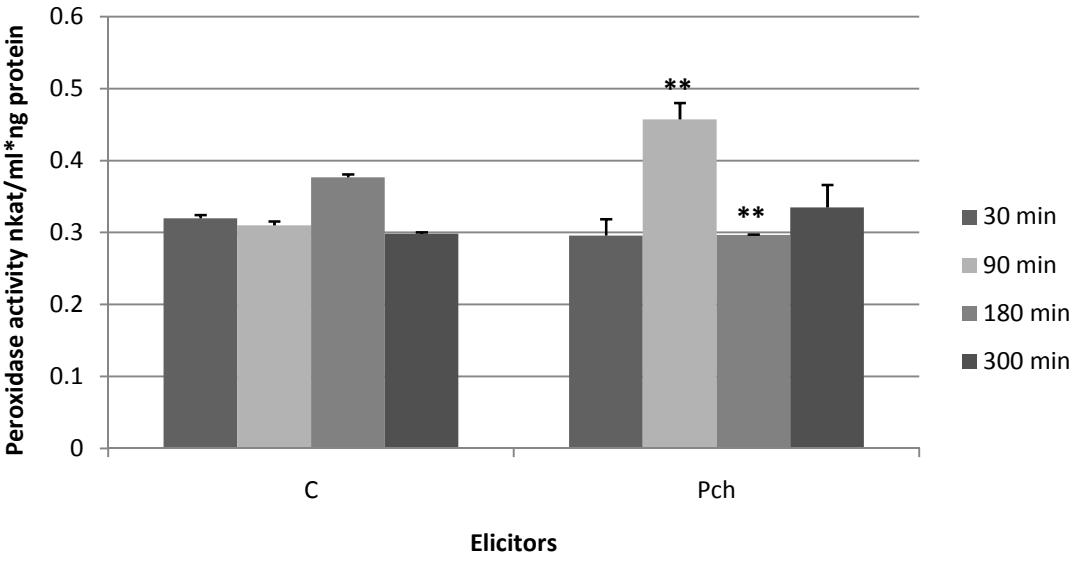

Figure 3.13 – Peroxidase activity (nkat/ml*mg protein), at 30, 90, 180 and 300 minutes after fungal elicitation of *V. vinifera* cv. Vinhão cell cultures. C – Control; *Pch* – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three replicates (experiment was repeated twice). Asterisks indicate significant statistical differences relative to control (** $p<0.01$).

Peroxidase activity is significantly higher than control 90 minutes after fungal elicitation and significantly lower 180 minutes after elicitation. These might indicate that peroxidase (along with other enzymes) plays a role in the oxidative burst: the increase in peroxidase activity 90 minutes after elicitation (around the time where ROS starts to accumulate in the second phase of the

oxidative burst) seems to indicate that peroxidase might play a role as ROS producer, as well as the lower peroxidase activity at 180 minutes (shortly before the second oxidative burst reaches its maximum) may contribute to ROS decrease by producing less ROS (lesser than control levels). However, the activity of peroxidase as ROS producer needs further confirmation by quantifying *Pch*-induced ROS in the presence of a peroxidase inhibitor.

ROS production by peroxidase was demonstrated in other plant systems upon fungal elicitation. It was shown in *Arabidopsis thaliana* elicited with a preparation of *Fusarium oxysporum* cell wall and in French bean cells in response to *Colletotrichum lindemuthianum* elicitation (Bindschedler *et al.*, 2006; Bolwell *et al.*, 1998). Also, in potato, all plant tissues exhibited an increase in peroxidase activity after infection with *Clavibacter michiganensis* (the causal agent of potato ring rot); in this system, higher peroxidase activity was correlated to resistance (Graskova *et al.*, 2004). Moreover, in grapevine, higher peroxidase activity was shown to positively correlate with *P. viticola* resistance (Kortekamp and Zyprian, 2003).

In plants, NADPH oxidase is the best studied font of stress-induced ROS, as analogous to a mechanism existing in animal cells (Bolwell *et al.*, 2002). DPI was proposed as a specific inhibitor of NADPH oxidase (Morré, 2002), and has been widely used in plant studies.

The profile of ROS accumulation induced by *Pch* elicitation in the presence of NADPH oxidase inhibitor (DPI) is shown in Figure 3.14.

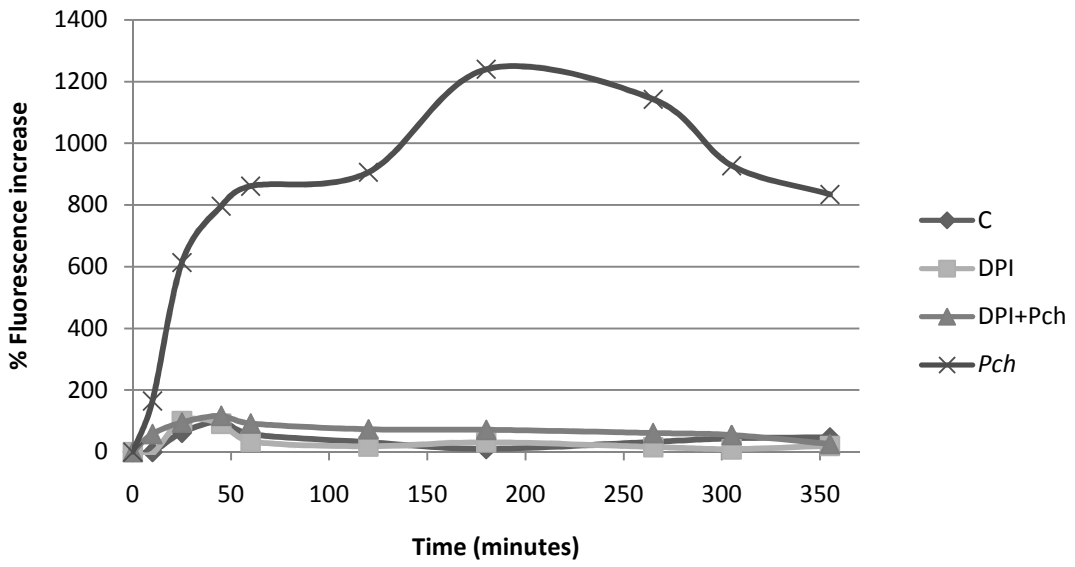

Figure 3.14 - ROS levels indicated by fluorescence increase (%) relative to 0 minutes (corresponding to fungal elicitation) in *V. vinifera* cv. Vinhão cell cultures. C – Control; DPI – DPI (10µM); DPI+*Pch* – DPI (10µM) + *Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results represent typical data obtained calculating % of fluorescence increase from the average of duplicates (experiment was repeated three times).

The results show that *Pch*-induced ROS accumulation is strongly inhibited. Because DPI is specific to NADPH oxidase, and peroxidase has been described as DPI-insensitive (Bindschedler *et al.*, 2006; Bolwell *et al.*, 1998), it seems that the majority of ROS produced in *V. vinifera* cv. Vinhão in response to *Pch* extract elicitation are derived from NADPH oxidase.

To further confirm these results, *Pch*-induced NADPH oxidase activity was measured at 30, 90 and 180 minutes after fungal elicitation and in the presence of DPI. Results are shown in Figure 3.15.

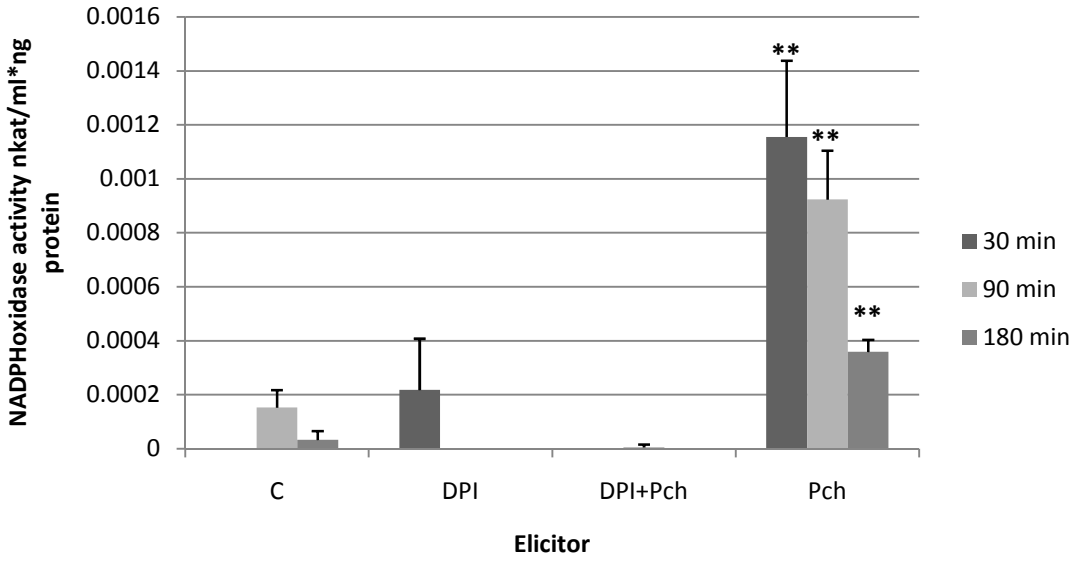

Figure 3.15 – NADPH oxidase activity (nkat/ml*ng protein), at 30, 90, and 180 minutes after fungal elicitation of *V. vinifera* cv. Vinhão cell cultures. C – Control; DPI – DPI (10µM); DPI+*Pch* – DPI (10µM)+*Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three replicates (experiment was repeated twice). Asterisks indicate significant statistical differences relative to control (** $p<0.01$).

Results show that *Pch* elicitation significanly increases NADPH oxidase activity when compared to control. Also, the presence of DPI completly inhibits *Pch*-induced NADPH oxidase, confirming the previous results.

NADPH oxidase-dependent oxidative burst has been reported in the litterature. In tobacco epidermal cells, the oxidative burst induced by cryptogenin (fungal elicitor from *Phytophthora cryptogea*) was inhibited by DPI (Allan and Fluhr, 1997). Also, the treatement of tomatto cells with race-specific elicitors of *Cladosporium fulvum* led to NADPH oxidase activation (Xing *et al.*, 1997), and ROS accumulation in cotyledons of *Mimosa pudica* elicited with ergosterol and chitosan was shown to be preceded by an increase in NADPH oxidase activity (Rossard *et al.*, 2006).

3.2.4 – Oxidative burst induced by *Pch* in *V. vinifera* cell cultures is dependent of Ca^{2+} influx

Pathogen elicitation induces several defence mechanisms in plants, from ion fluxes to phytoalexin accumulation. Ca^{2+} influx has been studied as one of the earliest components of defence response. To investigate the influence of Ca^{2+} influx on *Pch*-induced oxidative burst in *V. vinifera* cv. Vinhão cell cultures, the profiles of ROS accumulation in the presence of Ca^{2+} influx inhibitors were monitored. Results are shown in Figures 3.16 and 3.17.

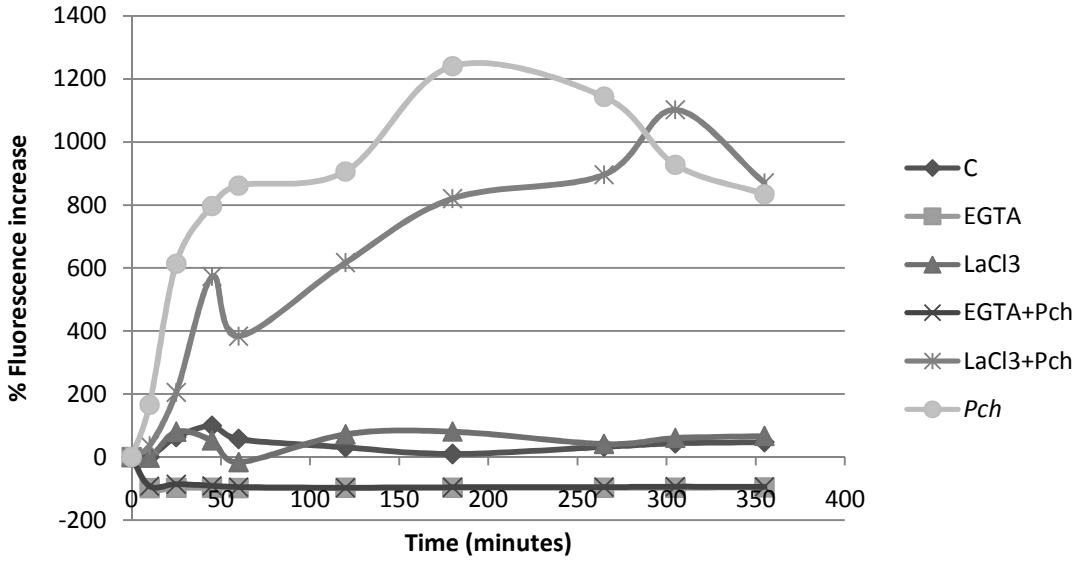

Figure 3.16 - ROS levels indicated by fluorescence increase (%) relative to 0 minutes (corresponding to fungal elicitation) in *V. vinifera* cv. Vinhão cell cultures. C – Control; EGTA - EGTA (5mM); $LaCl_3$ – $LaCl_3$ (100µM); EGTA+*Pch* – EGTA - EGTA (5mM)+*Pch* extract (0.5mg/ml); $LaCl_3$+*Pch* – $LaCl_3$ (100µM)+*Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results represent typical data obtained calculating % of fluorescence increase from the average of duplicates (experiment was repeated three times).

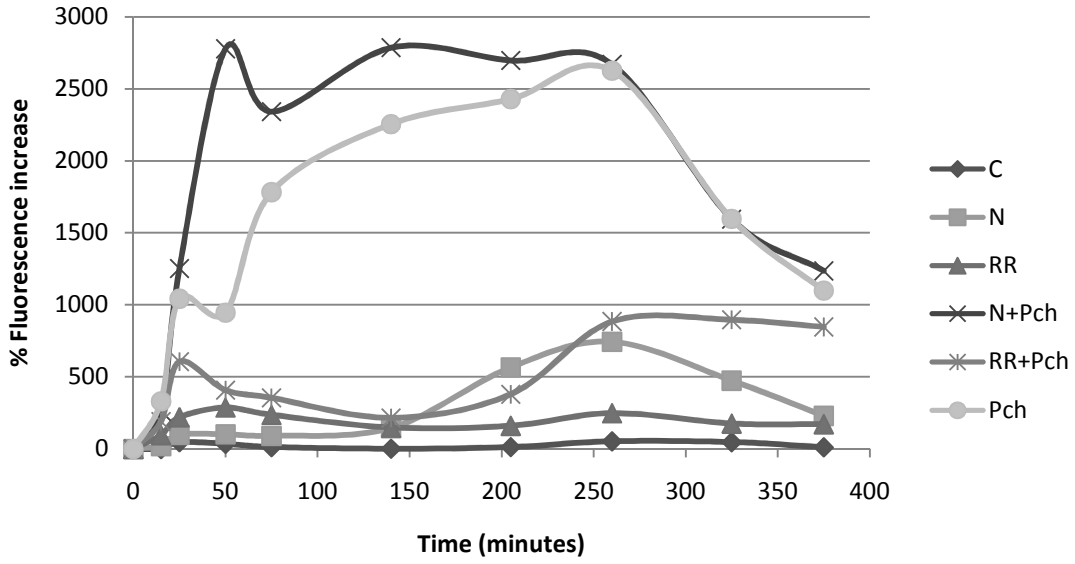

Figure 3.17 - ROS levels indicated by fluorescence increase (%) relative to 0 minutes (corresponding to fungal elicitation) in *V. vinifera* cv. Vinhão cell cultures. C – Control; N - nifedipine (100μM); RR - ruthenium red (100μM); N+*Pch* – nifedipine (100μM)+*Pch* extract (0.5mg/ml); RR+*Pch* – ruthenium red (100μM)+*Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results represent typical data obtained calculating % of fluorescence increase from the average of duplicates (experiment was repeated three times).

The results indicate that *Pch*-induced oxidative burst is dependent of Ca^{2+} availability and that ruthenium red-sensitive Ca^{2+} channels are strongly related to the oxidative burst.

The Ca^{2+} chelator EGTA completely inhibits ROS accumulation in *V. vinifera* cv. Vinhão cells, inclusively decreasing ROS levels below control (Figure 3.16). This suggests that ROS accumulation induced by *Pch* elicitation (and even at normal conditions) is heavily influenced by Ca^{2+} availability. EGTA was also shown to inhibit first and second phases of oxidative burst induced by *Phytophthora infestans* cell wall components in potato tubers (Kobayashi *et al.*, 2007); however, in *Catharanthus roseus* cells cultures, EGTA only partially inhibited fungal elicitor-induced ROS production (Zhao *et al.*, 2001), and in tobacco cells did not have any effect in inhibiting ROS accumulation in response to cadmium (Olmos *et al.*, 2003).

ROS accumulation is parcially inhibited in the presence of $LaCl_3$ (Figure 3.16). In micromolar concentrations $LaCl_3$ inhibits the majority of Ca^{2+}-permeable channels in the plasma membrane, depolarization- and hyperpolarization-activated and voltage independent channels, while leaving vacuolar channels unaffected (Kasparovsky *et al.*, 2003; White, 2000). $LaCl_3$ has been shown to block fungal cell wall elicitor ROS production (and subsequent phytoalexin production) in parsley cells (Zimmermann *et al.*, 1997). Also, $LaCl_3$ completely inhibit the oxidative burst induced in tobacco cells by exposure to cadmium (Olmos *et al.*, 2003), and, in *Pinus pinaster* cells suspensions $LaCl_3$ inhibits first and second phases of *Botrytis cinerea*-induced oxidative burst (Azevedo *et al.*, 2008). Interstingly, in tobacco cells, $LaCl_3$ completely inhibits ROS production induced by cryptogein, but only partially inhibits ergosterol-induced ROS accumulation (Kasparovsky *et al.*, 2003).

Ruthenium red strongly inhibits *Pch*-induced ROS accumulation (Figure 3.17). Ruthenium red inhibits plasma membrane depolarization-activated Ca^{2+} channels and tonoplast cyclic ADPribose-dependent channels (Kasparovsky *et al.*, 2003; White, 2000). This suggests that *Pch*-induced ROS accumulation is strongly dependent of these type of Ca^{2+} channels.

Interestingly, the presence of nifedipine does not inhibit ROS accumulation and seems to potentiate ROS accumulation induced by *Pch* extract (Figure 3.17). Nifedipine inhibits plasma membrane and tonoplast hyperpolarization-dependent Ca^{2+} channels (Kasparovsky *et al.*, 2003; White, 2000). The inhability of nifedipine to inhibit ROS accumulation in tobacco cells in response to cadmium was previously reported (Olmos *et al.*, 2003).

The importance of Ca^{2+} in plant defence is widely recognized. It has been shown that inhibition of Ca^{2+} influx leads to blockage of the downstream defence events oxidative burst and phytoalexin accumulation (Jabs *et al.*, 1997). Interestingly, a recent work described that bacteria are able to release molecules that chelate apoplastic Ca^{2+} and in this way suppress induced plant defences by blocking Ca^{2+} influx (Aslam *et al.*, 2008). Conversely, incompatible race-specific elicitors from *C. fulvum* were shown

to activate Ca^{2+}-permeable channels in tomato plasma membrane (Gelli *et al.*, 1997).

Overall, the results here presented, suggest that *Pch*-induced oxidative burst in *V. vinifera* cv. Vinhão cell suspensions is dependent of Ca^{2+} availability, and that the Ca^{2+} channel types from which Ca^{2+} influx is originated also are important to induce the oxidative burst (maybe because specific channels regulate the apropriate response to the stimulus). The fact that different elicitors induce different types of Ca^{2+} signatures have been described, and the possibility that different Ca^{2+} signatures encode the information to induce an appropriate response to the stimulus has been debated (Lecourieux *et al.*, 2002; Lecourieux *et al.*, 2006).

To further complement these results, NADPH oxidase activity in the presence of EGTA was measured at 30, 90 and 180 minutes after *Pch* elicitation (Figure 3.18).

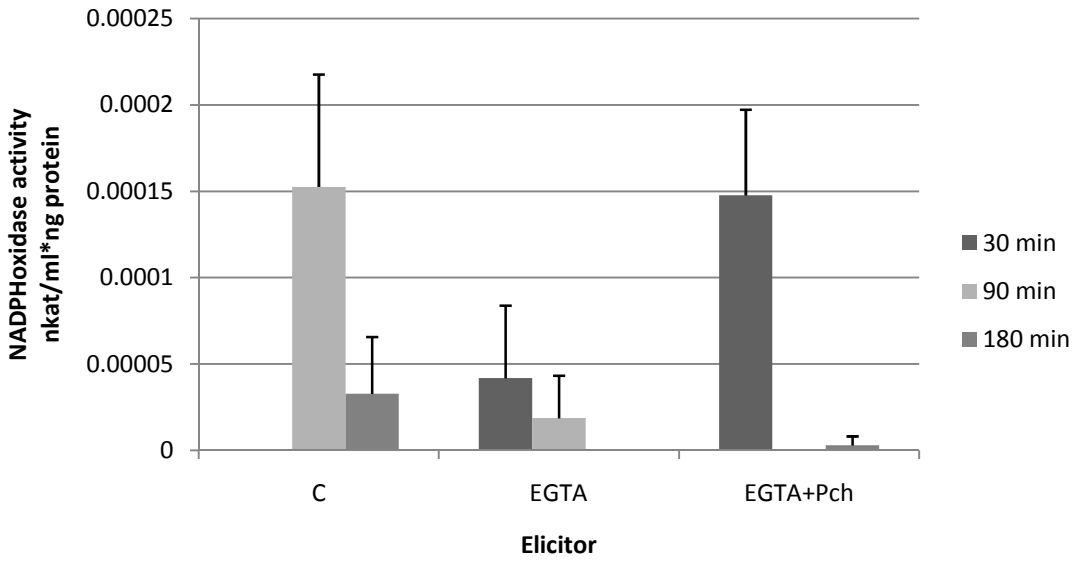

Figure 3.18 – NADPH oxidase activity (nkat/ml*ng protein), at 30, 90 and 180 minutes after fungal elicitation of *V. vinifera* cv. Vinhão cell cultures. C – Control; EGTA - EGTA (5mM); EGTA+*Pch* - EGTA (5mM)+*Pch* extract (0.5mg/ml); *Pch* – *Pch* extract (0.5mg/ml). Results are mean ± standard deviation of three replicates (experiment was repeated twice).

Results show that, in the presence of Ca^{2+} chelator EGTA, NADPH oxidase activity remains in control levels after *Pch* extract elicitation. This suggests that NADPH oxidase involved in *Pch*-induced oxidative burst is regulated by Ca^{2+}. In potato, NADPH oxidase was shown to be activated in a Ca^{2+} dependent manner, possibly through Ca^{2+}-dependent protein kinases (Kobayashi *et al.*, 2007). Also, it has been indicated that NADPH oxidase might be directly activated by Ca^{2+}, likely through Ca^{2+}-binding motifs (Sagi and Fluhr, 2006).

3.2.5 – Expression analysis of defence-related genes in *V. vinifera* cv. Vinhão cell cultures after *Pch* elicitation

The expression of seven grapevine defence-related genes was analysed in *V. vinifera* cv. Vinhão cell suspensions by semi-quantitative RT-PCR at 3, 12, 24 and 48 hours after *Pch* extract elicitation. The pattern of transcript accumulation of ACT, PR-6, PR-10, GLUC, CH3, PAL, STSY and LOX is shown in Figure 3.19.

ACT was checked for its suitability to be the housekeeping gene in this experiment. As Figure 3.19 indicates that ACT gene remains relatively stable throughout the experiment. Not showing major differences after *Pch* elicitation or along the time, grapevine ACT gene was considered suited to be the housekeeping gene, and was used to normalize gene expression in different samples. Expression of all other genes seems increased with *Pch* elicitation.

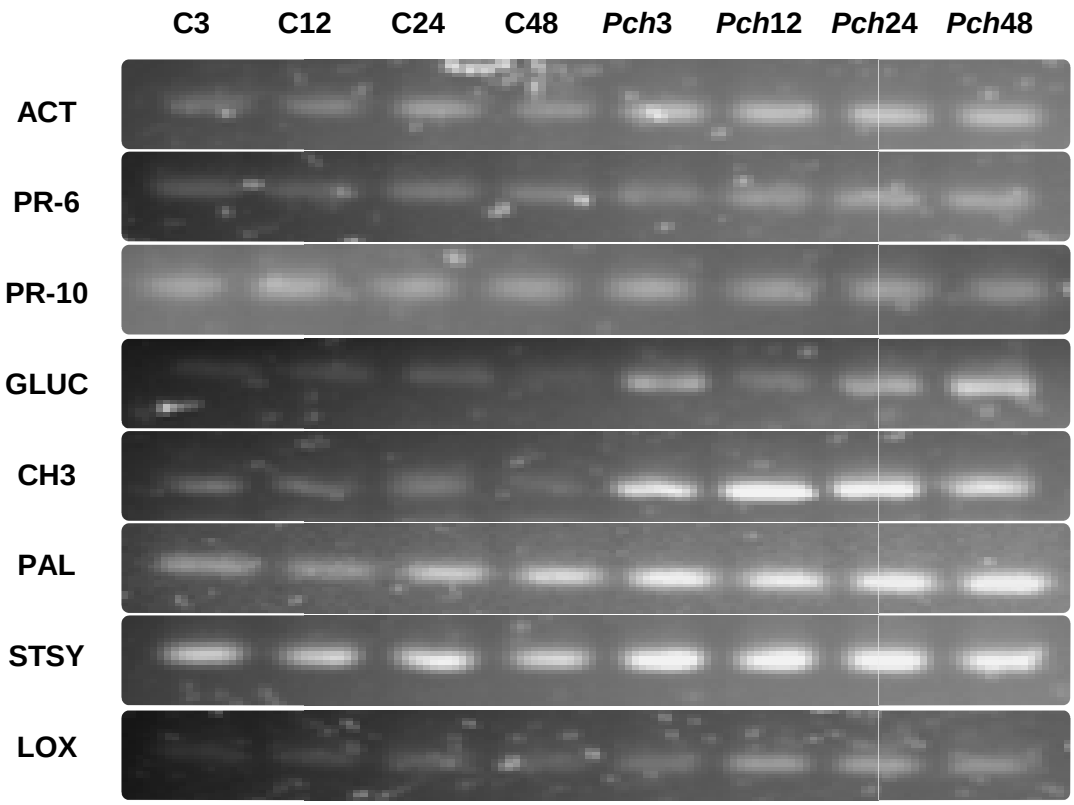

Figure 3.19 - Transcript accumulation of defence-related genes at 3, 12, 24 and 48 hours after *Pch* elicitation. C – Control; *Pch* – *Pch* extract (0.5mg/ml).

Housekeeping genes are usually constitutive genes involved in basic cellular functions (e.g. cell wall structure or primary metabolism) and, therefore, are supposed to indicate the rate of transcription of genes that are not altered by the experiment, as well as to be proportional to the amount of mRNA in analysis. In this way, housekeeping genes are used as internal controls that allow the normalization of differences between individual samples, since the amount of amplifiable RNA or cDNA is affected by amount and quality of starting material as well as by error introduced in RNA preparation and/or cDNA synthesis. (Al-Bader and Al-Sarraf, 2005; Reid *et al.*, 2006). Lately, the necessity of confirming the stability of previously assumed housekeeping genes have been stressed since some studies revealed that some of these genes might vary with experimental conditions (Al-Bader and Al-Sarraf, 2005; Reid *et al.*, 2006). For example, ACT gene was shown to be up-regulated during *V. vinifera* leaves-*P. viticola* interaction (Polesani *et al.*, 2008). Here, ACT expression remained stable under experimental conditions and, therefore, was considered fit to be used as housekeeping gene.

Figure 3.20 shows expression levels of PR-6, PR-10, GLUC, CH3, PAL, STSY and LOX; control samples of each time were defined as 1x expression of that time. Although all these genes are shown to be constitutively expressed in *V. vinifera* cv. Vinhão cell cultures (but with different intensities), *Pch* extract elicitation increased the transcription of all these genes and results suggest that these genes are regulated during the time course of infection.

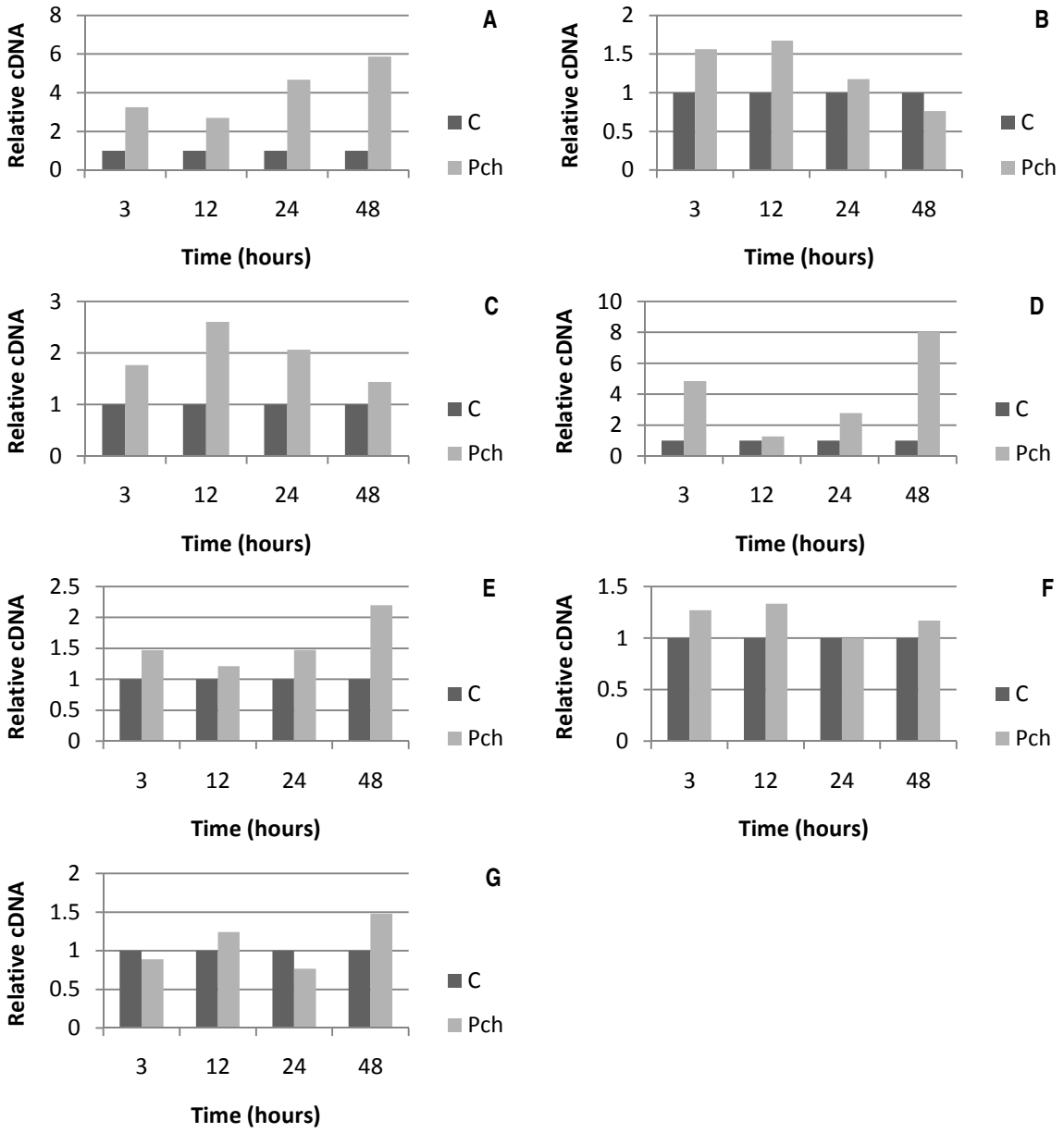

Figure 3.20 - Gene expression at 3, 12, 24 and 48 hours after *Pch* elicitation C – Control; *Pch* – *P. chlamydospora* extract (0.5mg/ml); A – PR-6; B – PR-10; C - GLUC; D – CH3; E - PAL; F - STSY; G - LOX. Control samples of each time was defined as 1x expression level. Each sample was normalized by actin gene expression. Results represent typical data obtained in two independent experiments.

PR-6, CH3, PAL, STSY and LOX show a biphasic pattern of induced expression, while PR-10 and GLUC induced expression peaks only once during the time of experiment.

PR-6, CH3 and PAL transcripts have a peak at 3 hours post elicitation followed by a second stronger increase from 24 hours on (Figure 3.20 A, D and E). STSY and LOX transcripts reach a peak at 12 hours post elicitation and show a new increase at 48 hours (Figure 3.20 F and G). PR-10 and GLUC transcripts seem to hit the highest level at 12 hours post elicitation decreasing from then on (Figure 3.20 B and C).

The expression of all these genes was already shown to be induced in *Vitis* in response to several stresses. However, the timings and patterns of induction vary with experimental system or elicitor used.

PR proteins are defined as *plant proteins that are induced in pathological or related situations* (van Loon *et al.*, 1994). Pathological conditions include all types of infection from microorganisms to herbivore attack, and related situations refer to situations that induce the same proteins as infection and include elicitors, chemicals as well as wounding (van Loon *et al.*, 1994). PR proteins have been organized in families and currently 17 classes of PR proteins have been described (Sels *et al.*, 2008). Here, the expression of PR-6, PR-10, PR-2 (β-1,3-glucanase) and PR-8 (class III chitinase) were studied.

Proteins belonging to PR-6 family include proteins with proteinase-inhibitor activity. Proteinase-inhibitors have been thought to play a role in plant defence by reducing the pathogen's capacity to use lytic enzymes to ensure penetration, to complete replication cycles or to feed by digesting host's' proteins (Sels *et al.*, 2008). In grapevine, PR-6 expression was shown to be increased in detached leaves subjected to UV-C irradiation (Bonomelli *et al.*, 2004), as well as PR-6 transcripts were shown to accumulate in leaves and berries infected with *B. cinerea* (Bézier *et al.*, 2002).

PR-10 proteins are thought to be involved in defence through their ribonucleolytic activity (Bantignies *et al.*, 2000; Moiseyev *et al.*, 1997). PR-10 induced expression was shown in grapevine in response to both biotic and abiotic stresses, with a monophasic pattern (as the results presented here). In grapevine leaves exposed to UV-C irradiation, PR-10 transcripts increased to a maximum at 24 hours post-irradiation and then decreased (Bonomelli *et al.*, 2004). Also, PR-10 transcripts accumulated in grapevine leaves after inoculation with both host and non-host pathogens (Kortekamp, 2006). Bacterial infection of *V. vinifera* leaves with *P. syringae* pv. *pisi* also induced increased PR-10 mRNA accumulation from 3 hours post-inoculation until reaching a maximum at 48 hours and decreasing thereafter, while infection with *Erysiphe necator* (the causal agent of powdery mildew) induces a continuous increase in PR-10 expression levels from 8 to 48 hours post-inoculation (Fung *et al.*, 2008; Robert *et al.*, 2001).

In plants, β-1,3-glucanase (PR-2) and class III chitinase (PR-8) are hydrolytic enzymes that degrade main components of pathogen's cell walls, β-1,3-glucans and chitin respectively, and are induced by infection with virus, bacteria or fungi (Kasprzewska, 2003; Leubner-Metzger and Meins Jr, 1999). These PR proteins act alone or combined in plant defence, either directly attacking pathogen's cell walls, or indirectly releasing cell wall-derived materials that will act as elicitors and induce defence responses (Kasprzewska, 2003; Leubner-Metzger and Meins Jr, 1999). In grapevine, leaves irradiated with UV-C showed an increase in β-1,3-glucanase and class III chitinase expression (Bonomelli *et al.*, 2004). Also, the *B. cinerea*-derived elicitors botrycin and cinerein up-regulated PR-2 and PR-8 in grapevine cell suspensions (Repka, 2006), *E. necator* infection led to PR-2 and PR-8 mRNA accumulation (Fung *et al.*, 2008), and infection of *Vitis* leaves with both *P. viticola* (host) and *Pseudoperonospora cubensis* (non-host) led to increased PR-2 expression (Kortekamp, 2006). In addition, class III chitinase expression was shown to be induced in grapevine leaves infected with *B.cinerea*, powdery mildew and *P. syringae* and in berries infected with *P. viticola* (Bézier *et al.*, 2002; Jacobs *et al.*, 1999; Robert *et al.*, 2002). Furthermore, in grapevine cells suspensions, class III chitinase

expression was increased in response to elicitation with yeast extract, suspension of live *P. syringae* and SA and related compounds (Busam *et al.*, 1997).

The biosynthesis of jasmonates (whose importance in defence signalling was discussed here in the previous sections 3.2.1 and 3.2.2) begins with the hydroperoxidation from linolenic acid by LOX (Creelman and Mullet, 1997), giving LOX an important role in defence. An important role in grapevine defence was already suggested for LOX given its up-regulation in leaves in response to UV-C irradiation (Bonomelli *et al.*, 2004), as well as in grapevine cells elicited with laminarin (a β-1,3-glucan derived from the brown algae *Laminaria digitata*) or challenged with the *B. cinerea*-derived elicitors botrycin and cinerein (Aziz *et al.*, 2003; Repka, 2006).

Since the production of stilbenic phytoalexins is one of the most common defence mechanisms in grapevine, it becomes obvious the importance of PAL (the first enzyme of the phenylpropanoid pathway) and STSY (the enzyme that synthesizes resveratrol using the cinnamic acid *p*-coumaroyl-CoA derived from the phenylpropanoid pathway) in grapevine defence (Jeandet *et al.*, 2002). In this way, PAL and STSY up-regulation has been reported in grapevine in response to several stresses. These genes were shown to be enhanced in *Vitis* leaves in response to UV-C irradiation (Bonomelli *et al.*, 2004), and leaves of *in vitro* grown grapevine plantlets showed a biphasic STSY mRNA accumulation in response to UV light (Borie *et al.*, 2004). Also, PAL and STSY expression was induced in grapevine leaves infected with *P. viticola* and in leaves and berries infected with *B. cinerea* (Bézier *et al.*, 2002; Kortekamp, 2006). In grapevine cells suspensions, PAL and STSY expression was shown to increase after elicitation with fungal cell walls, MeJ or *B. cinerea* derived elicitors (Melchior and Kindl, 1991; Repka, 2006; Repka *et al.*, 2001). Also, STSY transcripts were shown to be highly induced in *Vitis vinifera* leaves infected with the incompatible bacteria *P. syringae* (Robert *et al.*, 2001). Furthermore, a biphasic pattern of STSY expression was reported in grapevine cells cultures challenged with fungal elicitor and in grapevine leaves elicited with *E. necator* (Fung *et al.*, 2008; Wiese *et al.*, 1994). However, these two

authors reported different timings for STSY transcript accumulation: in cells cultures the first maxima of accumulation was at 3-5 hours after elicitation and the second was at 11-16 hours after elicitation, while in leaves the timings were delayed - a first maxima at 12 hours after inoculation followed by a second increase from 24 to 48 hours post inoculation (Fung *et al.*, 2008; Wiese *et al.*, 1994). Interestingly, the STSY kinetics of accumulation presented here are closer to the one reported in grapevine leaves. It has been suggested that the biphasic gene accumulation may allow the plant to adapt to a specific attack and modulate the defence response; the biphasic shape results of the consecutive expression of two types of genes: a first type quickly expressed after elicitation with a rapid degrading mRNA, followed by a second slowly expressed gene type (Wiese *et al.*, 1994).

Overall, the results here presented indicate that *V. vinifera* cv. Vinhão cells cultures are mounting a defence response elicited by the esca-associated fungus *Pch* since elicitation was able to induce the transcriptional activation of several genes commonly associated with plant defence. Therefore, *V. vinifera* cv. Vinhão cells cultures/*Pch* extract seems to be a fit model to study the molecular mechanisms involved in esca disease.

3.3 - Conclusions

In order to establish an *in vitro* model to study putative defence responses of grapevine to esca-related fungi, *calli* and cells suspension cultures were established from leaf explants of *V. vinifera* cv. Vinhão.

Pch extract elicitation of *V. vinifera* cv. Vinhão cells cultures led to an increased phenolic production, including the *de novo* production of the viniferin type compounds ε-viniferin-2-glucoside, ε-viniferin-glucoside and a polymer of two ε-viniferin molecules. Viniferins were already described as antifungal compounds. Also, the suspension cultures allowed the study of the effect of the signalling molecules SA and MeJ on defence. Particularly, MeJ showed to have potential in grapevine protection since it led to an increase in stilbenic and anthocyanin production; plus MeJ induced the *de novo* production of the same viniferin type compounds as *Pch* elicitation.

Besides, *Pch* extract elicitation of *V. vinifera* cv. Vinhão cells cultures resulted in a biphasic oxidative burst, typical of incompatible interactions. The influence of priming with the phytohormones SA and MeJ in the oxidative burst was also analysed. Although ROS accumulation seems to be potentiated in the first phase of the burst by SA priming, priming with both SA and MeJ seems to be obstructive to ROS accumulation during the second phase of the oxidative burst. Cells suspensions also allowed to study NADPH oxidase involvement in the oxidative burst, leading to the conclusion that this enzyme is the primary ROS font in this process. The putative role of catalase and peroxidase enzymes in the oxidative burst was also analysed. The results indicated that catalase activity is not altered by *Pch* elicitation and, therefore, other types of detoxifying mechanisms must be regulating

ROS burst. Regarding peroxidase, results indicated that this enzyme may play a role during the second phase of the oxidative burst. The influence of Ca^{2+} in the oxidative burst was also evaluated, and it was concluded that Ca^{2+} is necessary to oxidative burst occurrence and that the Ca^{2+} channel types from which Ca^{2+} influx is originated are also important to induce oxidative burst. Furthermore, Ca^{2+} availability also seems to be necessary for the activation of NADPH oxidase.

In addition, *Pch* extract elicitation of *V. vinifera* cv. Vinhão cells cultures was capable of inducing the expression of the defence-related genes PR-6, PR-10, GLUC, CH3, PAL, STSY and LOX.

Thus, *Pch* extract elicitation of cells cultures could induce several defence mechanisms (phytoalexin production, oxidative burst, Ca^{2+} signalling, NADPH oxidase activity and defence-related gene expression) indicating that *V. vinifera* cv. Vinhão cells suspensions could be an important tool to study esca disease, since they allow to selectively study host defence response to esca-related fungi without interference of external factors in a short period of time (2-3 weeks), leading to reliable results. Also, occupying a small space, cells suspension cultures make possible the analysis of a large number of culture flasks and/or cultivars. In conclusion, *V. vinifera* cv. Vinhão cells suspension cultures offer a simple, rapid and selective way to investigate the interaction between *Vitis* plants and esca-related fungi, therefore representing a valuable model to study esca disease.

CHAPTER 4 - ESCA AFFECTED

Vitis vinifera cv. Alvarinho LEAVES

In the previous page: illumination by Jean Bourdichon, from *Horae ad usum romanum – Grandes heurs d'Anne de Bretagne* (1503-1508), Tours – France; Bibliothèque nationale de France, Département des Manuscrits, Division occidentale (cote Latin 9474, folio 156). Accessed from Mandragore, base des manuscrits enluminés de la Bibliothèque nationale de France (http://mandragore.bnf.fr/html/accueil.html) on 12th January 2009.

4.1 - Introduction

The ability of plants to defend themselves of pathogen attack have long been noticed by humans and the use of some plants as pesticides has been reported since ancient times in Roman, Greek, Chinese and Indian works (Regnault-Roger and Philogène, 2008). Not surprisingly, the study plant defence metabolites has been of great interest to plant pathologists willing to unravel which compounds give resistant plants an advantage over pathogen infection and expecting that such insight could help to develop new strategies to protect susceptible plants. These defensive compounds result mainly from secondary metabolism and are highly diversified in chemical structure and among plant species. However, most phytoalexins belong to four classes: phenolics, acetylenics, terpenoids and nitrogen-containing compounds (Harborn, 1999).

Many studies on plant-pathogen interaction focused only in these classes of compounds in an attempt to understand the chemical response of plants to infectious agents. However, technical progress, of both analytical techniques and data analysis, led to an increasing number of studies of the whole metabolome resulting of such interactions, pursuing an improved understanding of defence response.

4.1.1 – Plant metabolomics

The term metabolome (defined as “total complement of metabolites in a cell”) was first used in the microbiology field, in a study analysing

Escherichia coli metabolism alterations in response to different growth conditions (Tweeddale *et al.*, 1998). Shortly after, metabolome analysis was recognized as an important and promising approach in plant biology (Tretheway *et al.*, 1999).

It is believed that the study of the metabolome can give more information on how a plant is responding to stress, instead of the study of genome, transcriptome or proteome. Although transcriptomics and proteomics can be important to understand the biological potential of an organism to interact with stressful conditions, it is known that mRNA increases are not always correlated to protein synthesis and, also, that synthesized proteins are not always enzymatically active. So, the changes on the metabolome are the ultimate result of the interaction with stress and, therefore, the more accurate information on how an organism is responding to stress (Bailey *et al.*, 2003; Sumner *et al.*, 2003). Hence, metabolomic studies have recently been applied in the field of plant biology to investigate plant responses to abiotic stresses (e.g. salinity, temperature, heavy metal), biotic stresses (plant-pathogen interactions) and to analyse substantial equivalence of genetic modified crops, to control food authenticity and quality and to complement genomic studies (Allwood *et al.*, 2008; Sanchez *et al.*, 2008; Shulaev *et al.*, 2008; Ward *et al.*, 2007).

Although the ultimate objective of metabolomics is the rapid and simultaneous identification and quantification of all metabolites present in a cell, tissue or organism, this goal is difficult to reach at present time. This is the main limitation of metabolomics and is related to the complex nature of the metabolome, with an enormous array of chemically diverse metabolites that occur in various concentrations ranging from pM to mM (Colquhoun, 2007; Sumner *et al.*, 2003).

Several analytical techniques have been used in plant metabolomics, mainly chromatographic methods like HPLC, or methods based in physical characteristics of metabolites such as molecular weight (MS) and resonance of magnetic nuclei (NMR spectroscopy). To obtain a more complete visualization of the metabolome both methods have been combined, given

that no single method can reach the ultimate goal of metabolomics and that every available method has its own limitations (Kim *et al.*, 2006; Sumner *et al.*, 2003; Verpoort *et al.*, 2007).

Recently, one-dimensional (1D) NMR is becoming the method of choice, at least as a first approach, in plant metabolomic studies. NMR advantages include simple and rapid sample preparation as well as rapid and automated measurement times. Moreover, NMR is non-destructive and non-selective, detecting a broad range of compounds, therefore giving a representative (although simplified) view of the metabolome. Furthermore, NMR spectra are highly reproducible and signals reflect molar concentrations allowing direct comparisons; also NMR spectra contain structural information about the metabolites detected. Particularly, ^{1}H-NMR spectroscopy is a good choice in plant metabolomic studies given the universal occurrence of protons in organic metabolites (Colquhoun, 2007; Kim *et al.*, 2006). The main disadvantages of this analytical method are the low sensitivity and spectra complexity and signal overlapping. However, these disadvantages can be overcome, at least in part, using two dimensional (2D) NMR techniques (Krishnan *et al.*, 2005; Ward and Beale, 2006) and, more recently, using chromatographic separation before NMR analysis (LC-NMR), often in tandem with more sensitive methods such as MS. In addition, increasingly sophisticated multivariate methods are employed to extract selected information from large sets of complex data such as NMR spectra.

Two-dimensional NMR techniques are essential for the identification of compounds to be made in complex mixtures. Several 2D NMR methods are available, including homonuclear and heteronuclear experiments that investigate correlations between nuclei of the same or different types, respectively. Total correlation spectroscopy (TOCSY) is a powerful homonuclear 2D experiment that describes all interactions in a spin system. Heteronuclear 2D experiments are also usually applied in parallel aiding compound assignment through the coupling information correlating for example proton (^{1}H) and carbon (^{13}C) nuclei (Fan and Lane, 2008; Ward and Beale, 2006).

The spectra resulting from plant extracts are very complex, comprising signals from many tens of small molecular weight compounds. In non-overlapped regions, 10-20 compounds may be easily identified by means of chemical shifts and coupling constants, and can be quantified by integration against an internal reference standard or by comparison with a reference method. However, the complexity (and great number of similar spectra obtained in multi-sample/multi-treatment experiments) necessarily require the use of multivariate statistical analysis in order to validate compound(s) variations responsible for differences between samples (Colquhoun, 2007; Kim *et al.*, 2006; Ward and Beale, 2006).

4.1.2 – PCA

PCA is a multivariate statistical method usually used in metabolomics. PCA describes the variance within the data while reducing the dimension of the data set: original variables are replaced by principal components (PCs), which are linear combinations of original variables. These PCs are calculated in decreasing order of importance, with the first PC explaining the maximum variability within the data, the second explaining the maximum of the remaining variability, and so on. Usually, the first two components are enough to separate different conditions. After calculation of the PCA model, the PCs can be graphically displayed as scores plots; these plots allow the observation of groupings within the data and also draw attention to outliers. Then, the plotting of PC loadings allows the detection of which variables are responsible for the groupings observed in scores plots (Colquhoun, 2007; Kim *et al.*, 2006; Ward and Beale, 2006).

Currently, several analytical techniques (such as NMR) followed by multivariate statistical analysis (namely PCA) are being used successfully in metabolomic studies and plant metabolomic studies became increasingly widespread in the last decade (Allwood *et al.*, 2008).

4.2 - Results and Discussion

4.2.1 - Differential phenolic production in leaves of *Vitis vinifera* cv. Alvarinho affected with esca disease evaluated by Principal Components Analysis

HPLC was used to evaluate the existence of differences between the phenolic profiles of *Vitis vinifera* cv. Alvarinho diseased leaves (dl- symptomatic leaves collected from infected boughs), apparently healthy leaves (ahl – asymptomatic leaves collected from infected boughs) and healthy leaves (hl – asymptomatic leaves collected from healthy boughs). HPLC methods have been used to analyse phenolic compounds of *Vitis* origin in leaves (Kolb *et al.*, 2001; Pastrana-Bonilla *et al.*, 2003), grapes (Cantos *et al.*, 2002; Landrault *et al.*, 2002), juices and wines (Betés-Saura *et al.*, 1996; Karagiannis *et al.*, 2000; Stecher *et al.*, 2001; Vitrac *et al.*, 2005), allowing the characterization of samples of different varieties, different disease stages or different processing stages in terms of amount and type of phenolic compounds present.

The chromatograms resulting from HPLC analysis of methanolic extracts of healthy, apparently healthy and diseased leaves are shown in Figure 4.1-A, B and C.

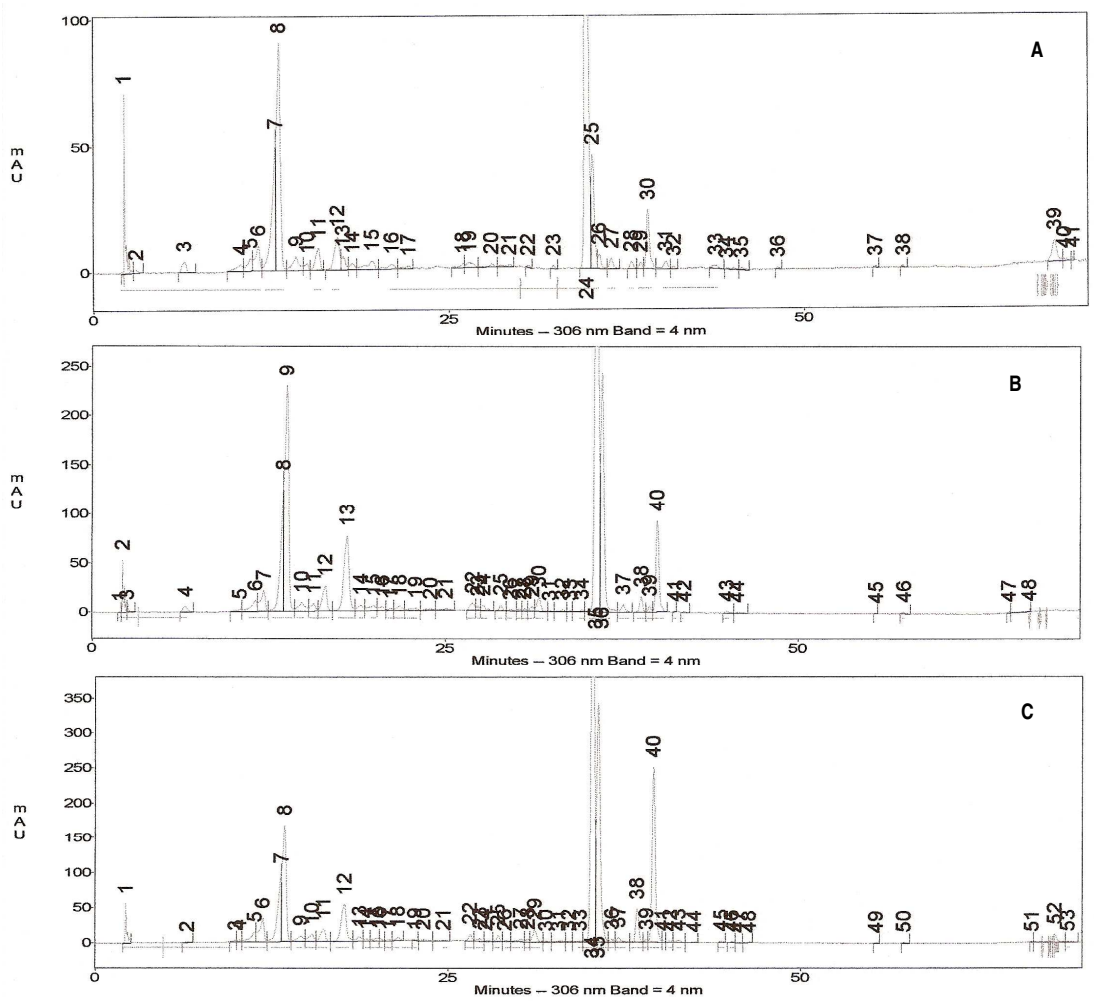

Figure 4.1 – Chromatograms of methanolic extracts of *Vitis vinifera* cv. Alvarinho leaves, 306nm. Representative of healthy (A); apparently healthy (B) and diseased (C) leaves.

No significant differences between the different types of leaves could be detected in the chromatograms by visual inspection alone. So, HPLC data was subjected to multivariate statistical analysis using PCA, with the purpose of visualizing any difference present between healthy, apparently healthy and diseased leaves phenolic profiles. To this date, there is no report of the use of multivariate statistical analysis applied to chemical data in the study of esca disease. This type of methodology has been used in the last few years in plant biology with several purposes. For instance, chromatographic analysis coupled with PCA has been used to identify the geographical origin

of a sample (Rauchensteiner *et al.*, 2005; Zhao *et al.*, 2005b), to distinguish samples from different parts of a fruit (Silva *et al.*, 2005), to distinguish/select individual genotypes of plants of the same species (Robinson *et al.*, 2005) and to assess quality control of products of natural origin such as jam (Silva *et al.*, 2006), honey (Arvanitoyannis *et al.*, 2005; Cordella *et al.*, 2003), cereals (Bonetti *et al.*, 2004), or liquors (Andrea *et al.*, 2003). Particularly, statistical multivariate analysis (namely PCA) applied to both instrumental and sensory data has been used recently to discriminate wines of different geographical region and/or variety (Arozarena *et al.*, 2000; Guggenbichler *et al.*, 2006; Kallithraka *et al.*, 2001; Silvertsen *et al.*, 1999).

Here, PCA analysis was applied to HPLC data with the objective of uncovering an eventual differential phenolic production due to esca disease and also to spot the major metabolites contributing to the differences between healthy, apparently healthy and diseased leaves. Before statistical analysis of HPLC data, all peaks detected were manually aligned to avoid biased results. To do this, chromatograms were examined to find the first common peak to all samples analysed, confirming by retention time and UV spectra. Once found this common peak, the retention time of all other peaks present in each chromatogram was divided by the retention time of the common peak of the matching chromatogram, in order to obtain the relative retention time of all compounds present in the chromatograms. All peaks detected in HPLC analysis were then aligned by their relative retention time and UV spectra were matched for all peaks to confirm that the alignment process was being performed accurately. Once all peaks were aligned, the peak areas were corrected by the amount of biomass extracted of the corresponding sample. The resulting data was analysed by PCA.

The application of PCA to HPLC data of 35 samples (4 diseased leaves were discarded as outliers) shows it is necessary 28 PCs to explain more than 99% of total variance. However, the PCs 1 and 2 are the best discriminating PCs, cumulatively accounting for 41% of total variance. The scores scatter plot resulting from the combination of these two PCs (Figure 4.2) clearly shows the separation of healthy, apparently healthy and diseased leaves. Furthermore, Figure 4.2 shows the apparently healthy

leaves approaching slightly the diseased leaves quadrant and thus indicating a path followed in PC space, probably signifying a change in phenolic production induced by the presence of pathogens, even before appearance of disease symptoms in these leaves.

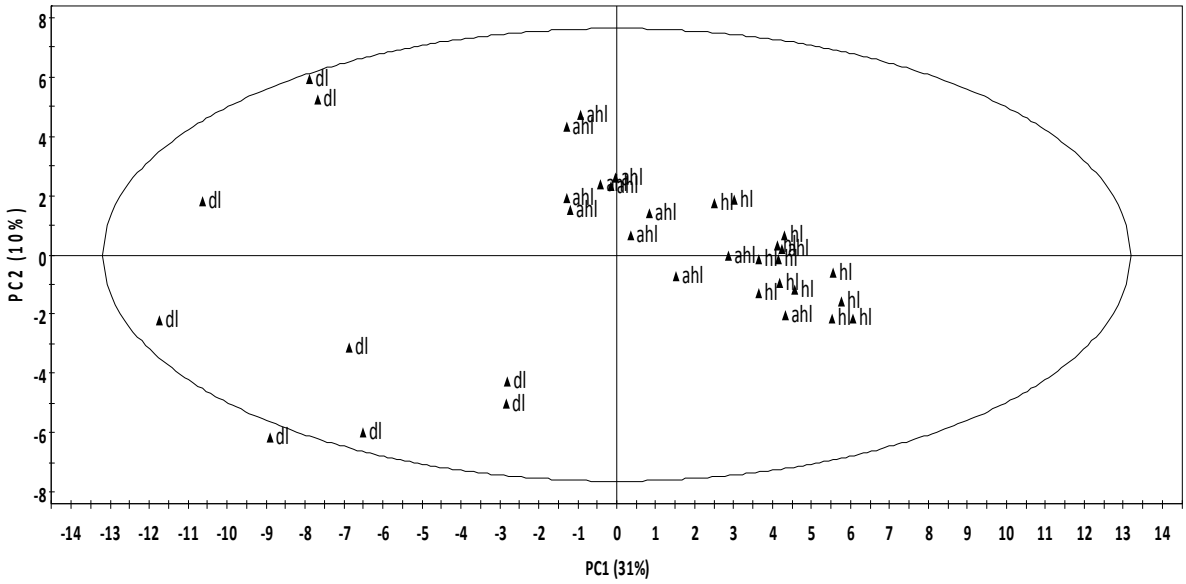

Figure 4.2 – PCA scores scatter plot, showing separation of healthy (hl), apparently healthy (ahl) and diseased (dl) *Vitis vinifera* cv. Alvarinho leaves along PC1. The ellipse represents the Hotteling T2 with 95% confidence.

The metabolites contributing the separation of the different types of leaves can be distinguished in a loadings plot (Figure 4.3), since the position of the samples in a given direction in a scores plot is influenced by the metabolites lying in the same direction in the loadings plot.

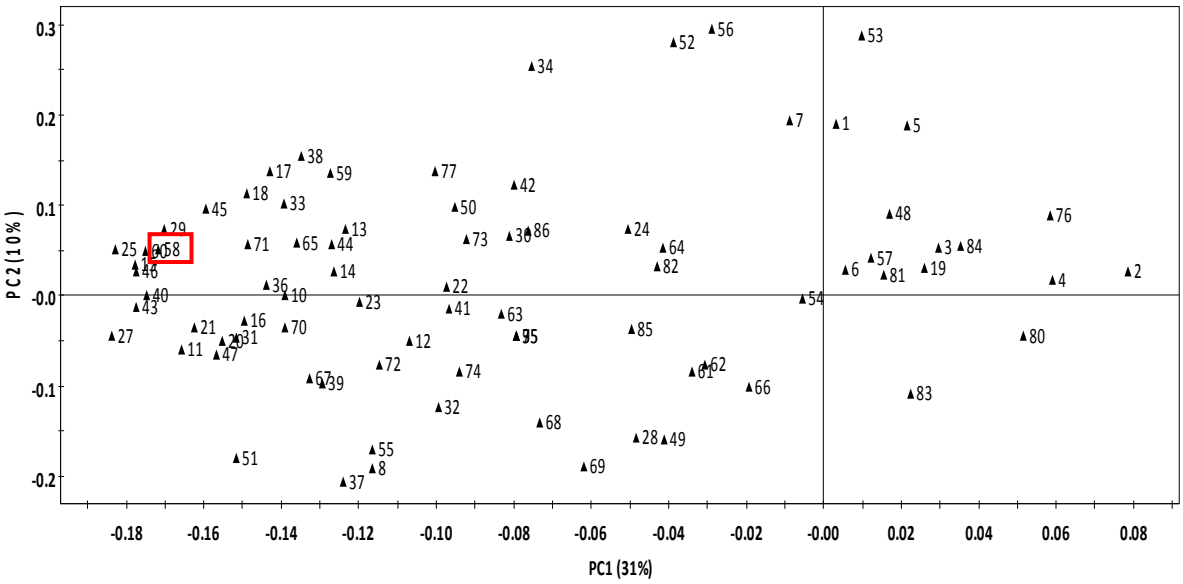

Figure 4.3 – PCA loadings scatter plot showing distribution of the compounds (named 1 to 86) detected by HLPC in *V. vinifera* cv. Alvarinho healthy and diseased leaves, as they correlate to PCs 1 and 2 extracted in PCA analysis. The red rectangle indicates the position of the metabolite selected and identified as kaempferol-3-glucoside.

Analysis of total phenolic production (Figure 4.4) shows that the amount of phenolics produced by both apparently healthy and diseased leaves is significantly higher than the amount produced by healthy leaves. A linear regression of these data shows that the phenolic production increases linearly from healthy to diseased leaves, with apparently healthy leaves occupying a medial position (r^2= 0.9924). As indicated by PCA analysis, it seems that a change in phenolic production is induced by the presence of pathogens, even before appearance of disease symptoms in these leaves.

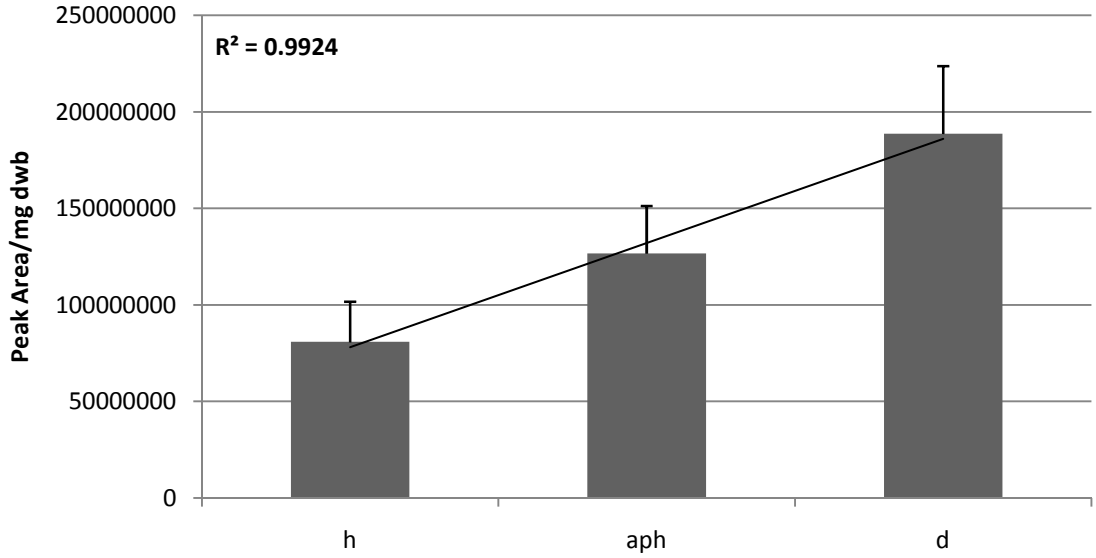

Figure 4.4 – Total phenolic production in healthy (hl), apparently healthy (ahl) and diseased (dl) *Vitis vinifera* cv. Alvarinho leaves. Line represents linear regression (r^2 displayed on chart).

Among the compounds strongly correlated with diseased leaves, one was selected (red rectangle in Figure 4.3) and identified as the flavonoid compound kaempferol-3-glucoside (Figure 4.5).

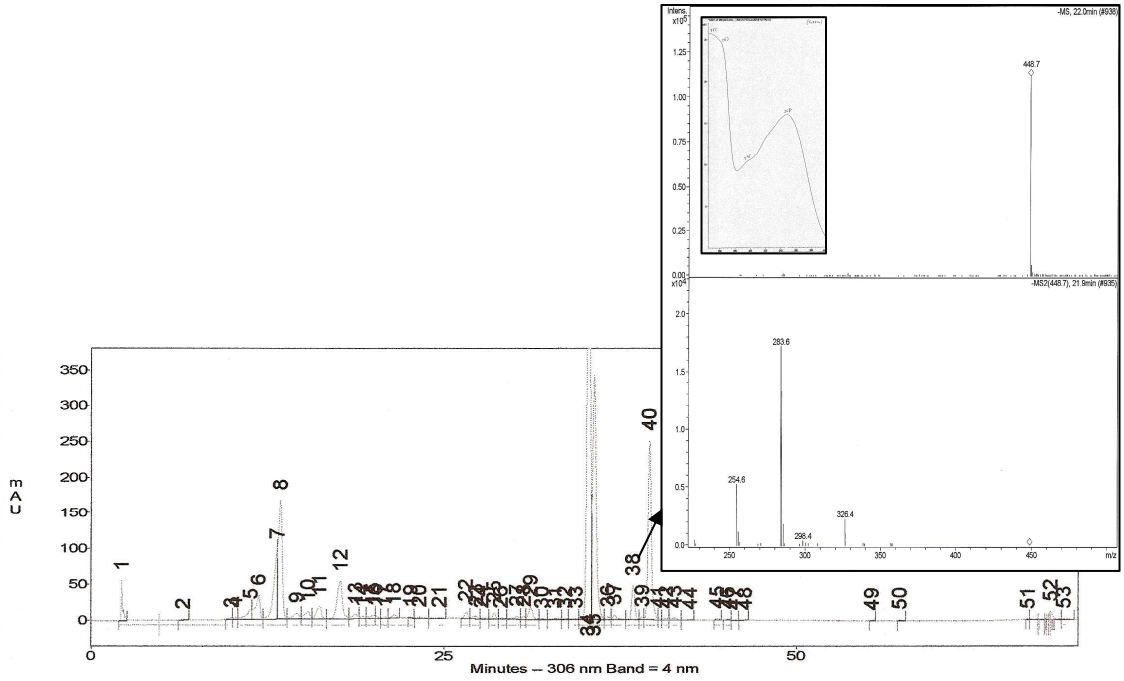

Figure 4.5 – Chromatogram representative of *Vitis vinifera* cv. Alvarinho diseased leaves; mass spectra and UV profile of peak 38 (corresponding to metabolite 58 in PCA) identified as kaempferol-3-glucoside.

Kaempferol-3-glucoside is strongly associated with diseased leaves and its amount increases from healthy to diseased leaves, with apparently healthy leaves containing an intermediate amount (Figure 4.6). Once again, the amount of this metabolite increases almost linearly from healthy to diseased leaves (r^2=0.9409).

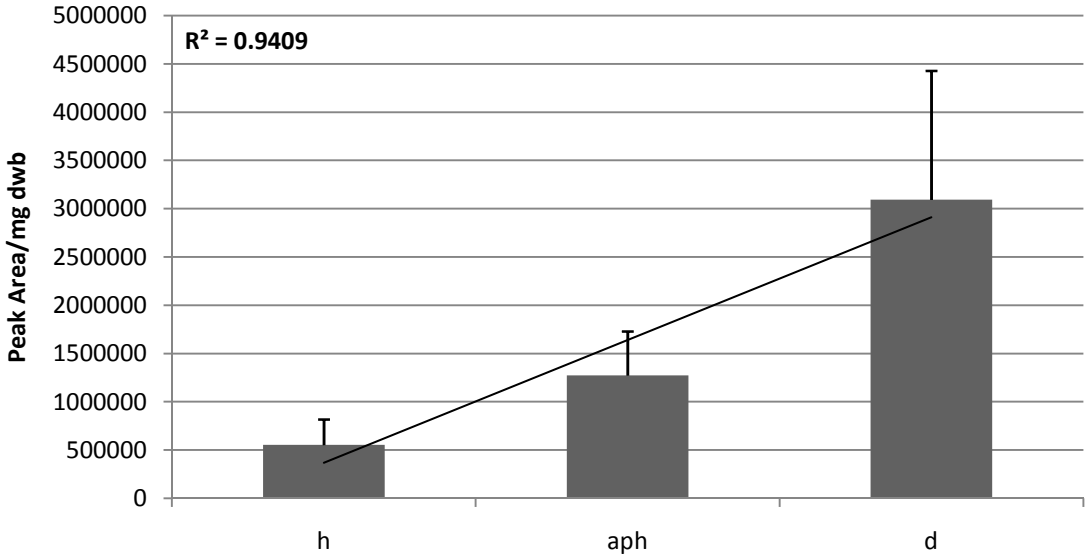

Figure 4.6 – Kaempferol-3-glucoside produced in healthy (hl), apparently healthy (ahl) and diseased (dl) *Vitis vinifera* cv. Alvarinho leaves. Line represents linear regression (r^2 displayed on chart).

The synthesis of phenolic compounds in response to stressful conditions is a recognized defence mechanism in plants. Flavonoids are a major group of phenolic compounds and centre of increasing interest due to their broad pharmacological activities. This is a group of secondary metabolites with great chemical diversity; compounds belonging to this group are known to play roles in normal conditions as well as in response to biotic and abiotic stresses. Flavonoids have been implicated in many plant processes and perform several different activities: modulation of growth and development,

attraction of pollinators, UV protection, metal toxicity protection, signals in symbiotic and in incompatible plant-fungus interaction, signals in plant-plant interactions, phytoanticipins and phytoalexins. (Dixon and Paiva, 1995; Harborn, 1999; Steikellner *et al.*, 2007; Treutter, 2006; Winkel-Shirley, 2001; Winkel-Shirley, 2002).

Several flavonoids including the compound kaempferol-3-glucoside – here identified and shown to be strongly correlated to diseased leaves – were detected by HPLC in strawberry and its amount was shown to be augmented in response to increases in growth temperature or carbon dioxide levels (Wang *et al.*, 2003; Wang and Zheng, 2001). In addition, these flavonoids were shown to be affected by the cultural system used, increasing when strawberries were cultivated in hill plasticulture system (Wang *et al.*, 2002). Flavonoid accumulation was also shown to be part of early defence response to *Xanthomonas* bacterial infection of cotton and to fungal infection of lupin (Bednarek *et al.*, 2003; Dai *et al.*, 1996). Specifically in grapevine, exposure to solar radiation was shown to induce accumulation of flavonoids, including kaempferol-3-glucoside, in both berry skin and pulp (Pereira *et al.*, 2006; Spayd *et al.*, 2002). Also, flavonoids and other phenolic compounds accumulate in grapevine calli in response to *Plasmopara viticola* fungal infection, with higher accumulation in resistant varieties implying that induced flavonoids might play a role in grapevine resistance to this fungus (Dai *et al.*, 1995b). Furthermore, antifungal activity of kaempferol and other flavonoids was demonstrated *in vitro* (Padmavati *et al.*, 1997).

Regardless of the great accumulation of phenolic compounds in diseased leaves, the plant could not avoid the appearance of symptoms. This may happen because activation of defence responses was too late (therefore confirming the susceptibility of Alvarinho variety to esca disease). It was already suggested that resistant and susceptible varieties have the same genetic patrimony or are able to activate the same defence responses that enable the plant to cope with fungal infection; nevertheless, susceptible varieties seem to be slower in the activation of defence mechanisms, while resistant varieties activate defence mechanisms rapidly and intensively upon fungal recognition, enabling them to cope with infection (Dai *et al.*, 1995a;

Figueiredo *et al.*, 2008). The absence of symptoms in apparently healthy leaves, despite the presence of fungi of the esca complex, together with an accumulation of phenolic compounds significantly higher than the amount present in healthy leaves, might indicate that these parts of the plant are mounting a defence response in time to cope with the infection. It is well established that attacked plants can synthesize and release secondary metabolites that act as chemical signals; the perception of these signals by non-attacked parts of the plant or by neighbouring plants will elicit the activation of defence responses, therefore preparing them to sustain a possible attack (Gawronska and Golisz, 2006). It is possible that this kind of signalling is occurring in esca infected *Vitis vinifera* cv. Alvarinho plants and allowing that parts of the plant remain asymptomatic. Nonetheless, the confirmation of these hypotheses requires further studies. Almost certainly, the defence mechanisms to esca would be better understood after detailed metabolomic analysis (to identify possible defence metabolites and putative signal molecules) and transcriptomic studies comparing resistant and susceptible varieties.

4.2.2 – Metabolomic analysis of *V. vinifera* cv. Alvarinho leaves showing esca disease symptoms using 1D and 2D NMR spectroscopy and multivariate data analysis

As the metabolic profile is the ultimate result of the plant response to an imposed stress, the study of the metabolome might bring a better understanding of the plant's defence mechanisms to cope with stressful conditions. In addition to the previously analysed phenolic profile (section 4.2.1), the overall metabolic profile of *V. vinifera* cv. Alvarinho leaves showing esca symptoms (diseased leaves – dl) and asymptomatic leaves (healthy leaves – hl) were compared. The extracts of 10 healthy and 10 diseased leaves were analysed by ^{1}H-NMR; their resulting spectra, ranging from 0 to 8.5 ppm, are presented in Figure 4.7. Given the high signal

intensity of the sugar region (3.2-6 ppm), enlargements of aromatic (6-8.5 ppm) and aliphatic (0-3.2 ppm) regions are shown for better visualization.

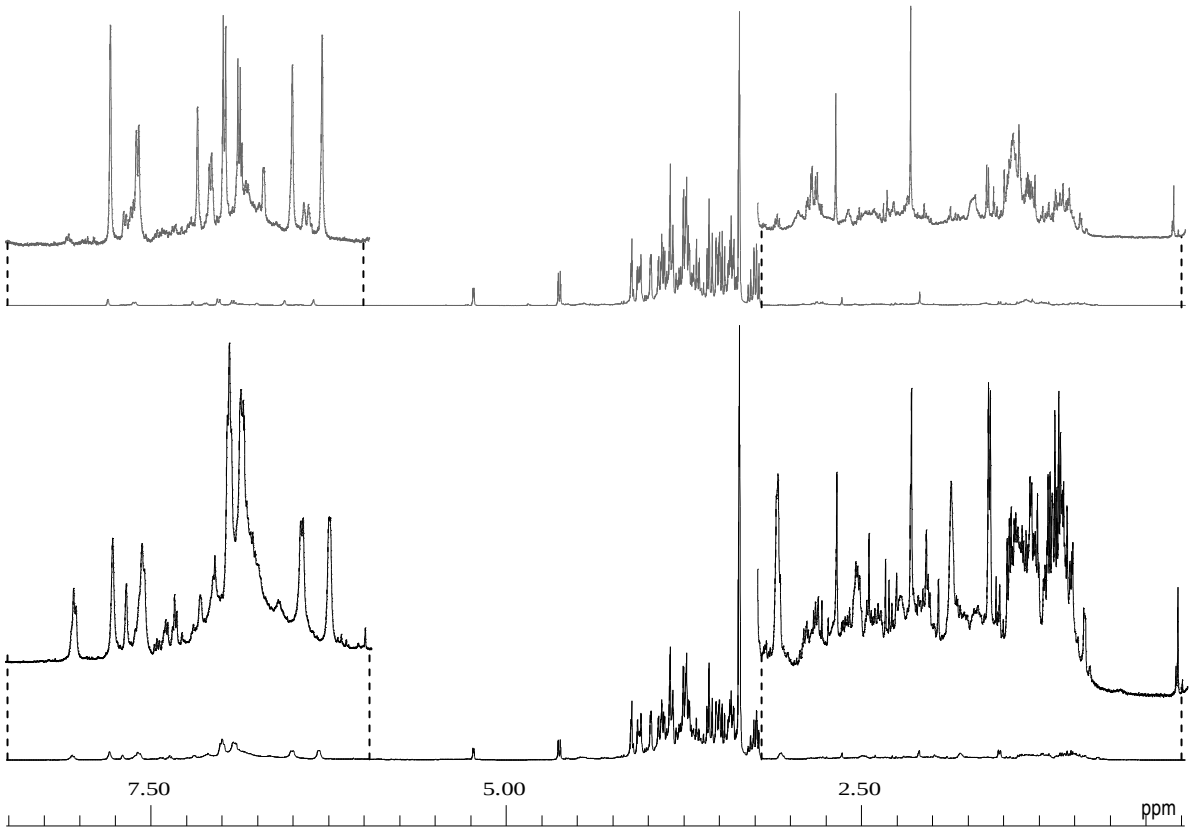

Figure 4.7 – ^{1}H-NMR spectra of *V. vinifera* cv. Alvarinho leaves (0-8.5 ppm), with enlargements of the aromatic (6-8.5 ppm) and aliphatic (0-3.2 ppm) regions. Healthy leaves (grey); diseased leaves (black).

From visual inspection of Figure 4.7 it is clear that the metabolic profile of diseased leaves is different from the one of healthy leaves. In particular, it is evident an increase in the aromatic region and the appearance of broad signals of large molecules (polyphenols) in the spectra of diseased leaves. This was expected since the synthesis of phenolic compounds in response to stressful conditions is a recognized defence mechanism in plants. Also, this increase of phenolic compounds in diseased leaves was previously described in the preceding section (4.2.1).

However, further interpretation including assignment of observed peaks is difficult due to spectral complexity and extensive overlapping of signals. To overcome the problems in the 1D ^{1}H-NMR spectra, 2D NMR techniques are

used, not only to overcome spectral congestion but also to improve spectral resolution and indicate which peaks belong to the same molecule (Ward and Beale, 2006). So, extracts of diseased and healthy leaves were subjected to TOCSY and ^{1}H-^{13}C correlation experiments. Spectra resulting from these two techniques are presented in Figures 4.8 and 4.9.

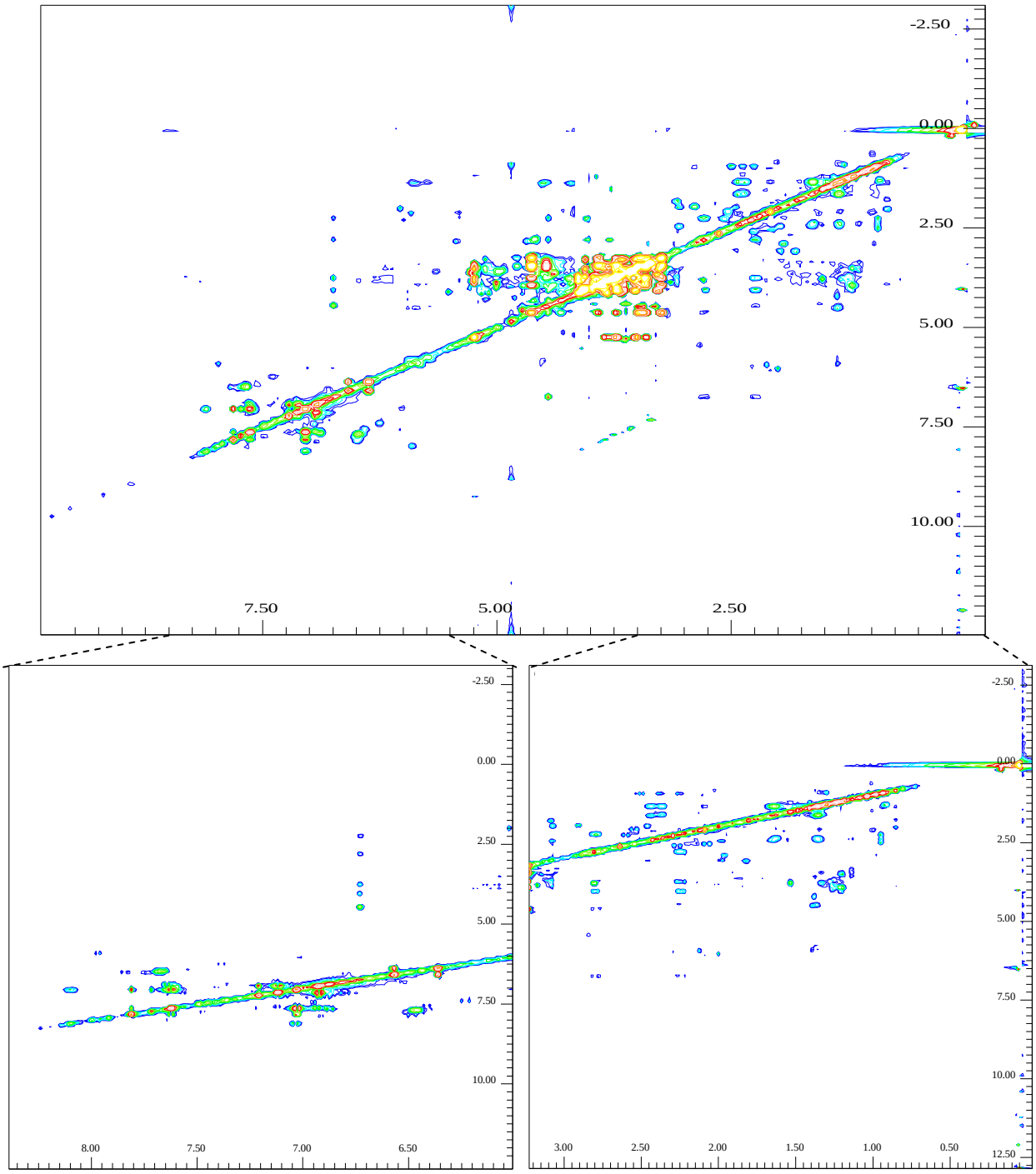

Figure 4.8 – TOCSY spectrum representative of *V. vinifera* cv. Alvarinho leaves (0-8.5 ppm), with enlargements of the regions 5.5-8.5 ppm and 0-3.5 ppm.

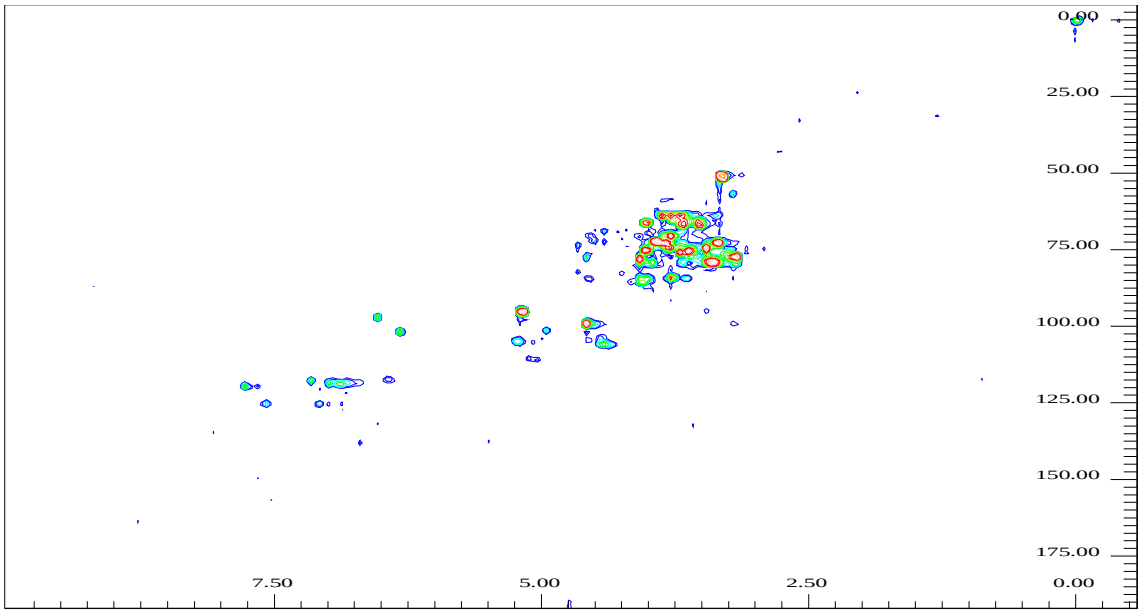

Figure 4.9 – ^{1}H-^{13}C correlation spectrum representative of *V. vinifera* cv. Alvarinho leaves (0-8.5 ppm).

By making possible to identify correlations between peaks, the results of TOCSY experiments enabled the identification of several spin systems. Additionally, the combination of 1D and 2D NMR techniques allowed the assignment of some peaks by comparison to available spectra. A complete list of the spin systems identified - assigned and unassigned (named A to R) - is given in Table 4.1. In the aliphatic (0-3.2 ppm) and sugar (3.2-6 ppm) regions the assignment of various peaks was possible by comparison to available libraries (Chemistry Department of Aveiro University). In the aliphatic region signals from amino acids (alanine and GABA) and organic acids (lactic, acetic, pyruvic and succinic acids) are identified. The signals identified in the sugar region arise from ascorbic and tartaric acids, methanol, inositol, fructose and α- and β-glucose. Finally, in the aromatic region (6-8.5 ppm), assignment was made by comparison to literature (Figueiredo *et al.*, 2008). Signals arising from shikimic acid, quercetin-3-*O*-glucoside and caffeic acid are identified. However, many signals remain unassigned due to signal overlap and/or small peak intensity. Further assignments require more sophisticated experiments (such as LC-NMR) that could increase sensitivity and reduce signal overlap.

Table 4.1 – ^{1}H chemical shifts, proton multiplicities and coupling constants of spin systems identified in *V. vinifera* cv. Alvarinho leaves.

	Compound	Chemical shift (ppm)	Multiplicity	J (Hz)	Compound	Chemical shift (ppm)	Multiplicity	J (Hz)
Assigned	Alanine	CH_3 1.53	d	7.2	Tartaric acid	4.65		
		α-CH 3.76	q		Methanol	CH_3 3.36	s	
	GABA	γ-CH_2 3.07	t	7.5	Inositol	C5H 3.29		
		α-CH_2 2.45	t	7.3		C4H/C6H 3.62		
		β-CH_2 1.99	m	7.4		C2H 4.06		
	Acetic acid	CH_3 2.09	s			C1H/C3H 3.53		
	Ascorbic acid	C4H 4.56			α-glucose	C1H 5.23	d	3.5
		C5H 3.86				C5H 3.85		
		$C6H_2$ 3.68				C3H 3.74		
	Lactic acid	CH_3 1.38	d	6.6		C2H 3.52		
		α-CH 4.20				C4H 3.41		
	Pyruvic acid	CH_3 2.41	s		β-glucose	C1H 4.63	d	7.9
	Shikimic acid	C7H 2.24				C6H 3.92		
		C7H' 2.80				C5H 3.74		
		C5H 3.76				C3H 3.49		
		C6H 4.06				C4H 3.40		
		C4H 4.47				C2H 3.24		
		C3H 6.73			Fructose	C3H 4.11		
	Succinic acid	CH_2 2.64	s			C5H 4.06		
	Caffeic acid	6.47				4.02		
		7.67				C4H 3.99		
		6.92	d	8.1		C6H 3.84		
		7.12				3.81		
		7.20				3.80		
	Quercetin-3-*O*-glucoside	7.03	d	8.4		3.69		
		7.62	d	8.8		3.67		
		7.80				3.57		
		6.36				3.60		
		6.57						
Unassigned	A	0.92			I	1.36		
		1.32				1.64		
	B	0.85				2.41		
		2.03			J	2.80		
		1.78				3.79		
		1.37			K	2.10		
	C	0.95				1.00		
		2.51			L	1.36		
		2.38				1.64		
		2.27			M	2.27		
	D	1.14				2.60		
		3.36			N	5.89		
		3.48				7.97		
		3.59			O	6.23		
	E	1.21				7.39		
		3.52			P	6.41		
		3.94				7.52		
	F	1.24			Q	6.48		
		3.90				7.84		
	G	1.82			R	7.04		
		3.08				8.09		
	H	1.97						
		2.91						

In order to statistically confirm the differences between the metabolic profiles of healthy and diseased leaves, and to unravel the peaks mainly responsible for those differences, the 1D ^{1}H-NMR spectral data was subjected to PCA multivariate statistical analysis.

A scores plot of the first two PCs calculated by PCA considering the entire spectra (0-8.5 ppm) is presented in Figure 4.10 and shows a clear separation of healthy (hl) and diseased (dl) leaves, along the PC1 (which accounts for 81% of total variance). The noteworthy proximity of duplicates (corresponding to 2 leaves of each grapevine), almost overlapping, is an indication of the high reproducibility of the technique. However, when analysing the corresponding PC1 loadings plot (Figure 4.11) with the purpose to find which peaks are contributing to the discrimination between leaves, the discriminating signals lie mainly in the sugar region, with the high signal intensity of this region masking possible discriminating signals in the aliphatic and aromatic regions. Therefore, in order to avoid masking the contribution of minor metabolites, PCA was performed in the 3 regions of the spectra separately.

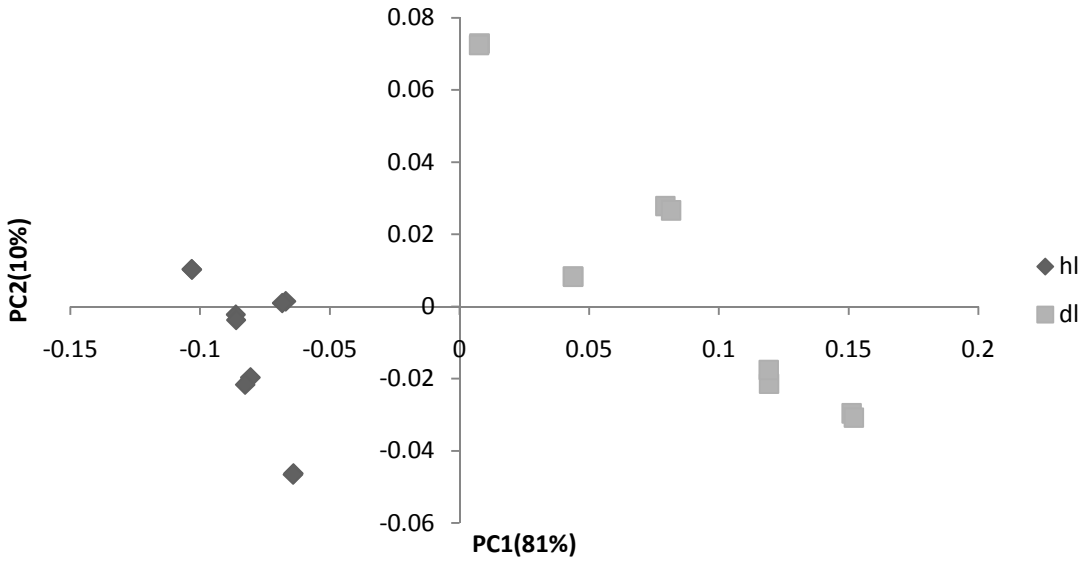

Figure 4.10 – PCA scores scatter plot obtained when analysis considers the whole spectra, showing separation of healthy (hl) and disease (dl) leaves by PC1.

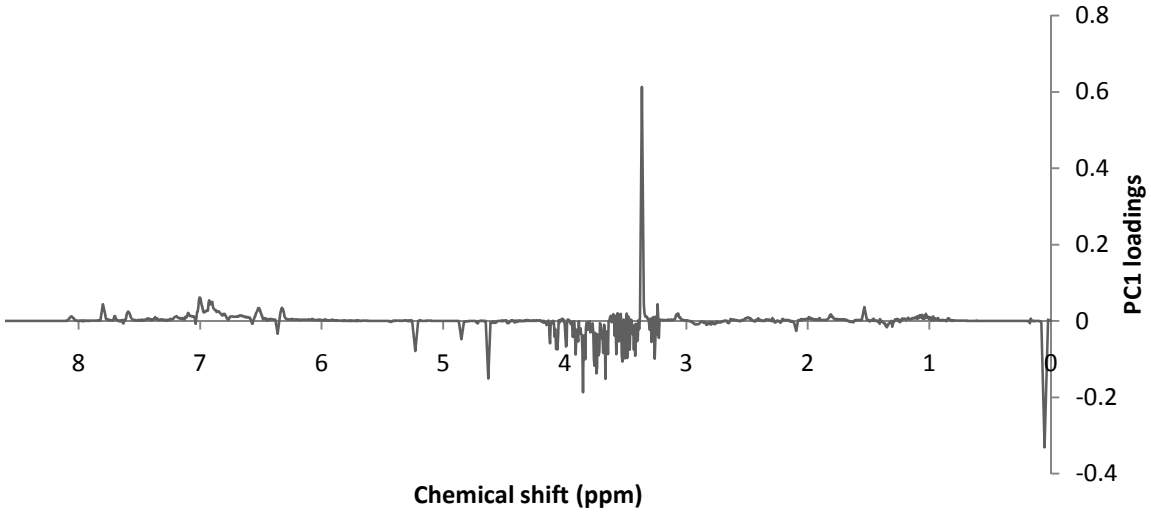

Figure 4.11 – PCA loadings scatter plot of PC1, obtained when analysis considers the whole spectra.

The scores plot of PC1 and PC2 calculated by PCA of the aliphatic region (Figure 4.12) shows separation of healthy (hl) and diseased (dl) leaves along PC1, which accounts for 55% of total variance. Again, the closeness of duplicates is noteworthy.

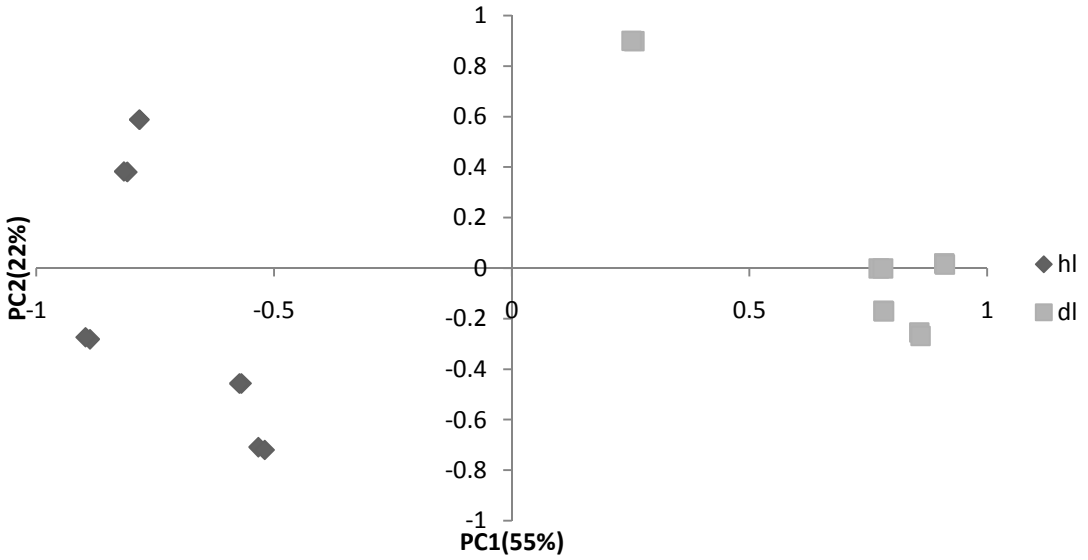

Figure 4.12 - PCA scores scatter plot obtained when analysis considers only the aliphatic region of the spectra, showing separation of healthy (hl) and disease (dl) leaves by PC1.

The interpretation of the corresponding PC1 loadings plot (Figure 4.13), together with visual inspection of the spectra (Figure 4.14), indicates the main compounds/peaks contributing to the discrimination of healthy and diseased leaves (by indicating which compounds/peaks are more abundant in each type of leaves). The compounds that separate healthy and diseased leaves in the aliphatic region are alanine, GABA, shikimic, acetic and pyruvic acids, unknown peaks at 1.35, 1.81 and 1.90 and 0.82-1.15 ppm (Table 4.2).

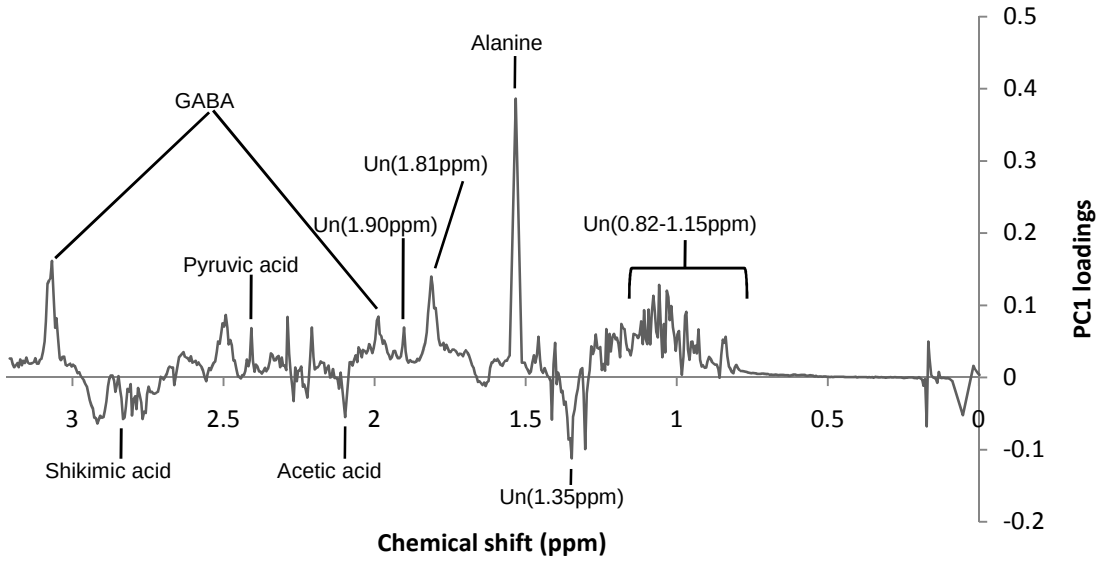

Figure 4.13 - PCA loadings scatter plot of PC1, obtained when analysis considers only the aliphatic region of the spectra.

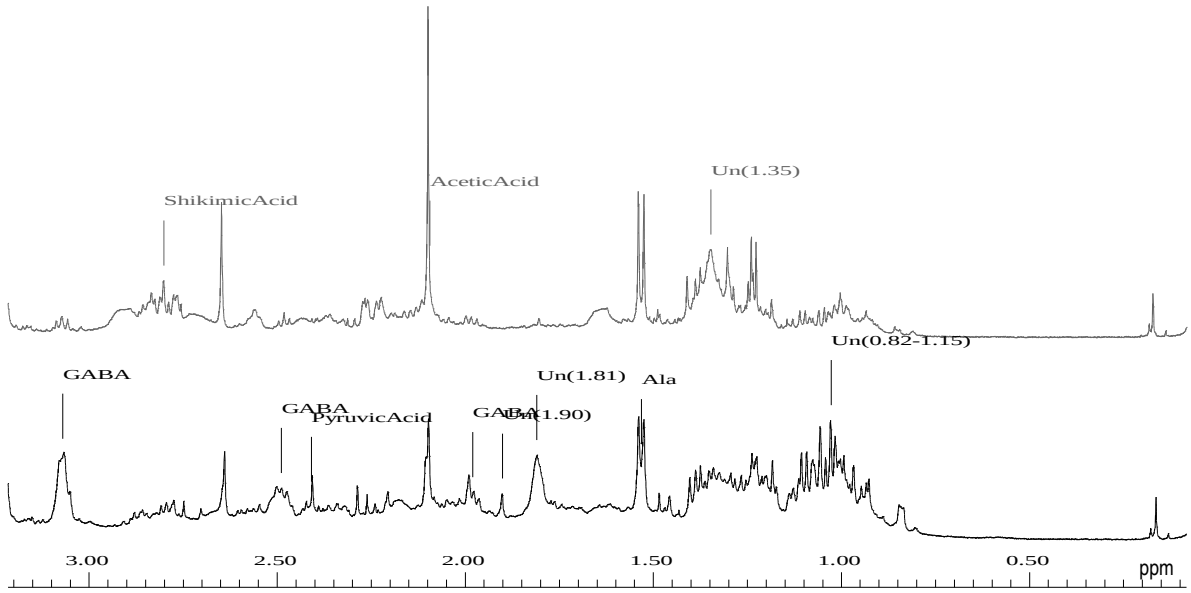

Figure 4.14 - ^{1}H-NMR spectra (aliphatic region 0-3.2 ppm) of *V. vinifera* cv. Alvarinho leaves, with compounds/peaks characteristic of healthy leaves (grey) and diseased leaves (black) annotated.

The scores plot of the first two PCs calculated by PCA of the sugar region (Figure 4.15) shows separation of healthy (hl) and diseased (dl) leaves along PC1, which accounts for 86% of total variance. Once more, the closeness of duplicates is worth mentioning.

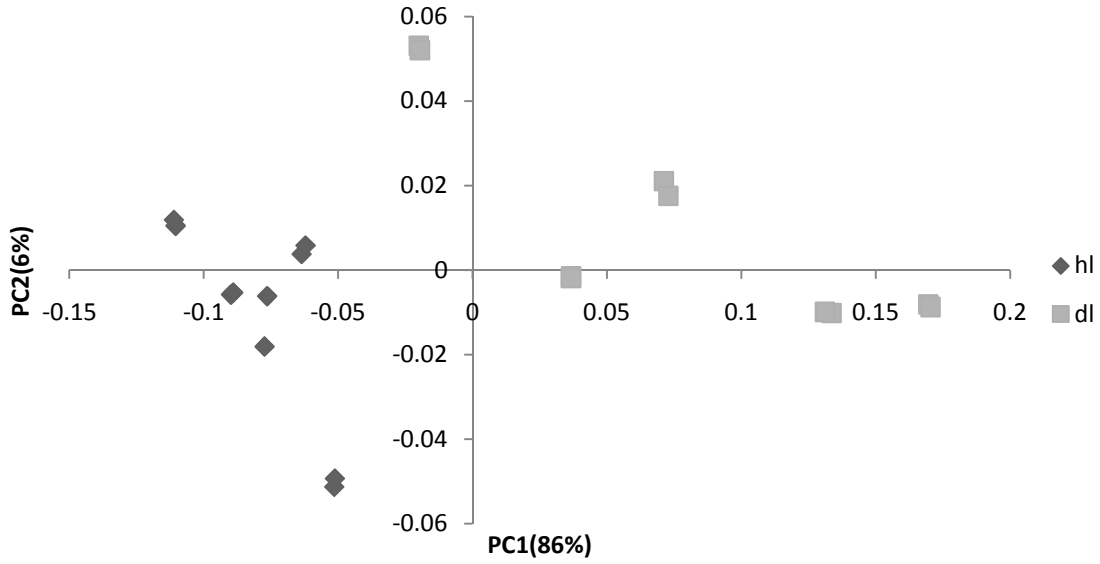

Figure 4.15 – PCA scores scatter plot obtained when analysis considers only the sugar region of the spectra, showing separation of healthy (hl) and disease (dl) leaves by PC1.

The interpretation of the corresponding PC1 loadings plot (Figure 4.16), together with visual inspection of the spectra (Figure 4.17), indicates that the compounds separating healthy and diseased leaves in the sugar region are α- and β-glucose, fructose, shikimic acid, unknown peak at 4.85 ppm and methanol (Table 4.2).

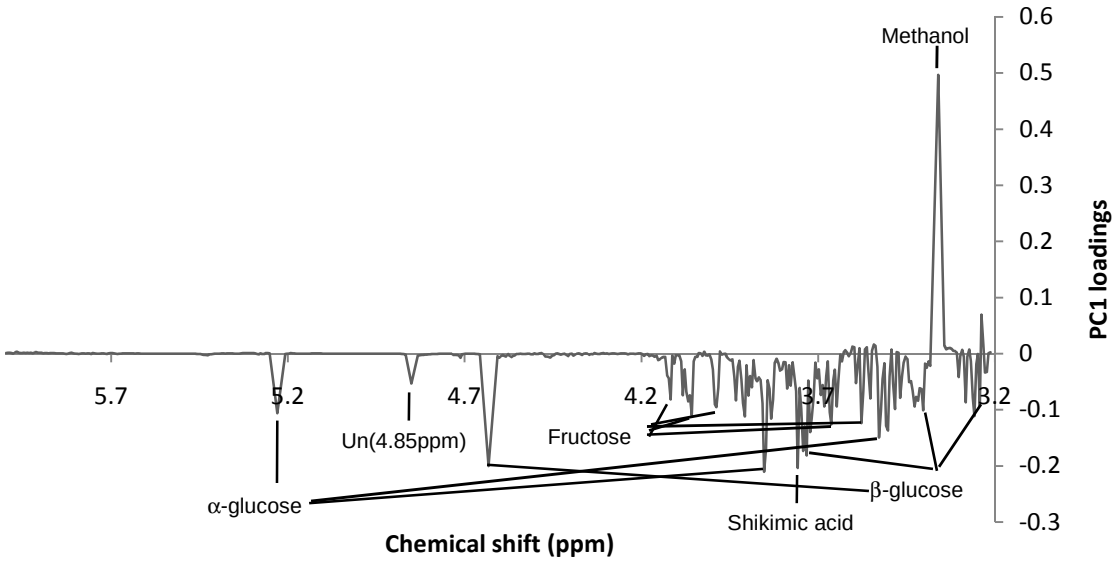

Figure 4.16 - PCA loadings scatter plot obtained when analysis considers only the sugar region of the spectra.

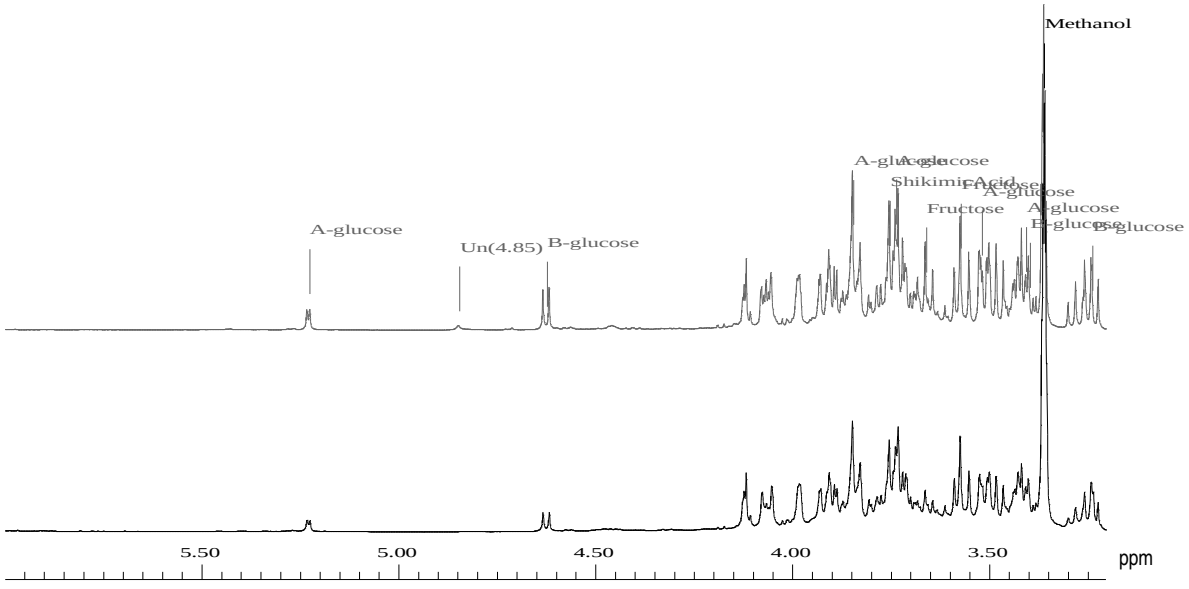

Figure 4.17 - ^{1}H-NMR spectra (sugar region 3.2-6 ppm) of *V. vinifera* cv. Alvarinho leaves, with compounds/peaks characteristic of healthy leaves (grey) and diseased leaves (black) annotated.

The scores plot of PC1 and PC2 calculated by PCA of the aromatic region (Figure 4.18) shows that PC1, which accounts for 74% of total variance,

clearly separates healthy (hl) and diseased (dl) leaves. The proximity of duplicates is striking here, leading to complete overlap. This figure also shows that a duplicate of healthy leaves is far-off from the healthy leaves cluster; this is due to the presence of a signal at 6.81 ppm in these 2 leaves that is absent in all other leaves.

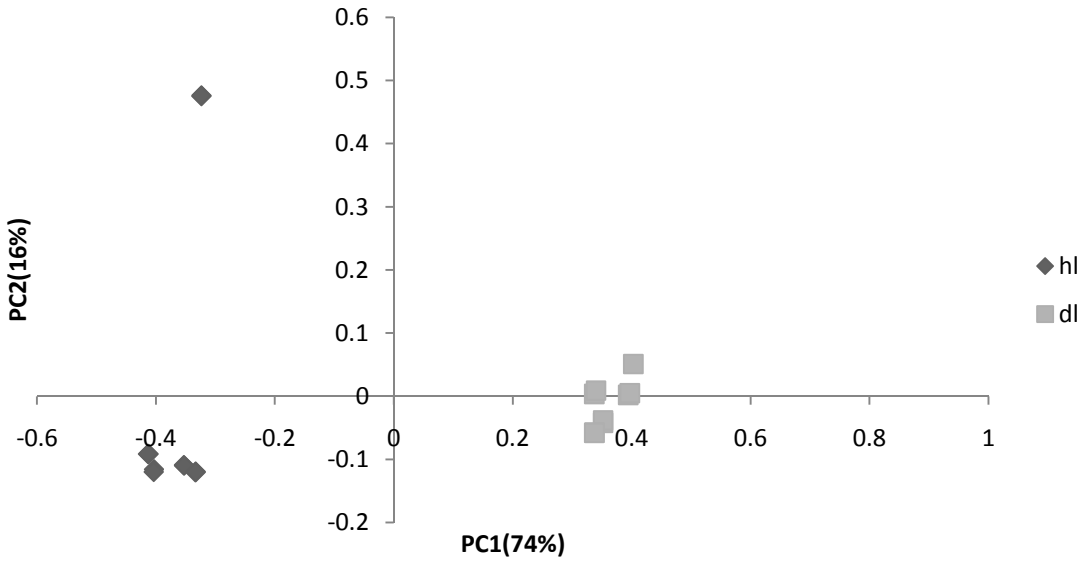

Figure 4.18 - PCA scores scatter plot obtained when analysis considers only the aromatic region of the spectra, showing separation of healthy (hl) and disease (dl) leaves by PC1.

The analysis of the corresponding PC1 loadings plot (Figure 4.19), together with visual inspection of the spectra (Figure 4.20), indicates that the compounds/peaks that discriminate healthy and diseased leaves in the aromatic region are quercetin-3-*O*-glucoside, caffeic acid and unknown peaks at 6.77, 8.05, 7.70, 7.58, 6.99, 7.09, 6.89-9.93, 6.52 and 6.32 ppm (Table 4.2).

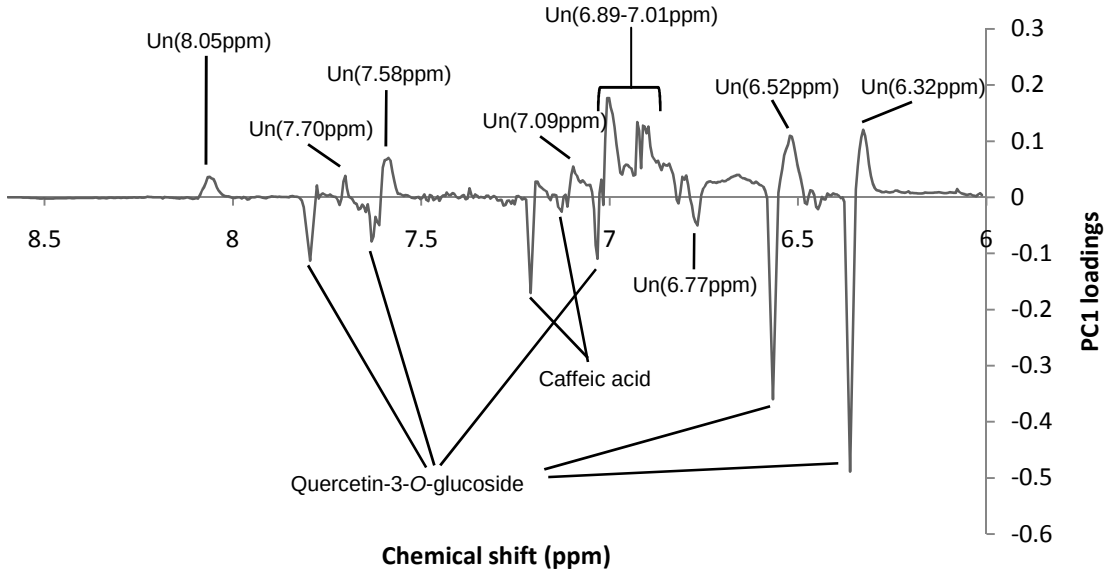

Figure 4.19 - PCA loadings scatter plot obtained when analysis considers only the aromatic region of the spectra.

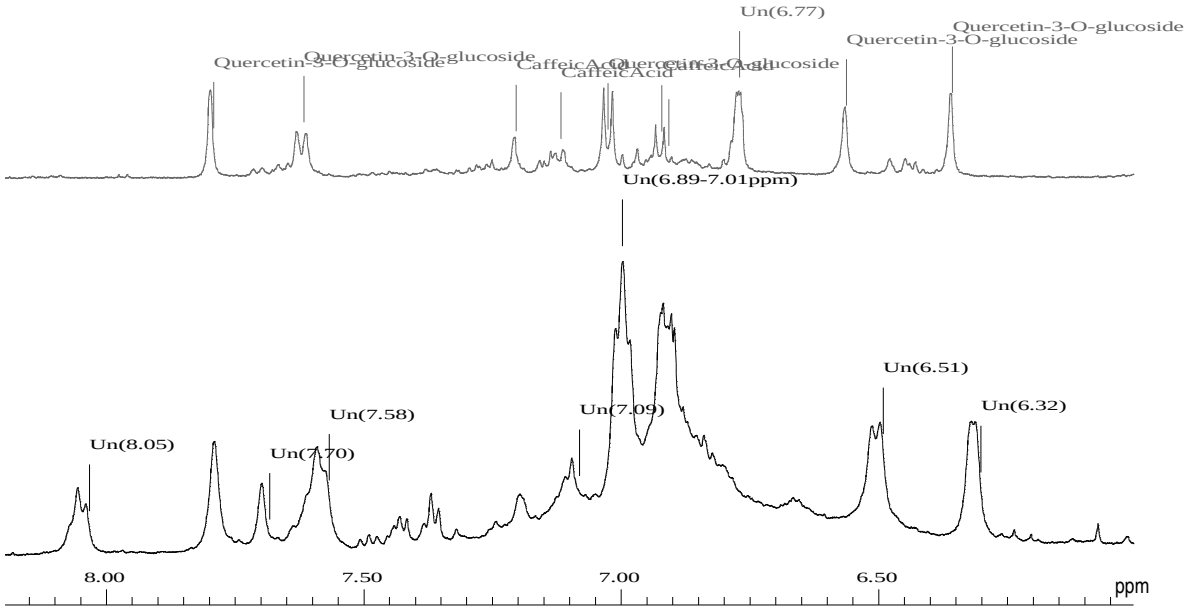

Figure 4.20 - ^{1}H-NMR spectra (aromatic region 6-8.2 ppm) of *V. vinifera* cv. Alvarinho leaves, with compounds/peaks characteristic of healthy leaves (grey) and diseased leaves (black) annotated.

Table 4.2 – Discriminating compounds/peaks of *V. vinifera* cv. Alvarinho healthy (hl) and diseased (dl) leaves, presented as increased or decreased in dl leaves.

Decreased in dl	Increased in dl
Un(1.35ppm)	Un(0.82-1.15ppm)
Acetic acid	Alanine
Shikimic acid	Un(1.81ppm)
α-glucose	Un(1.90ppm)
β-glucose	GABA
Fructose	Pyruvic acid
Shikimic Acid	Methanol
Un(4.85ppm)	Un(6.32ppm)
Quercetin-3-*O*-glucoside	Un(6.52ppm)
Caffeic acid	Un(6.89-7.01ppm)
Un(6.77ppm)	Un(7.09ppm)
	Un(7.58ppm)
	Un(7.70ppm)
	Un(8.05ppm)

Taken together, the results seem to indicate that the metabolism of diseased leaves is flowing towards the production of phenylpropanoid compounds (overview in Figure 4.21). When compared to healthy leaves, the diseased leaves metabolism appears to be characterized by active glycolysis (given the reduction of glucose and fructose and the increase of the final product pyruvate) with carbon dislocating through the shikimic acid-phenylpropanoid pathway (given the decrease in shikimic acid level and the increase of phenolic compounds - aromatic ^{1}H-NMR signals).

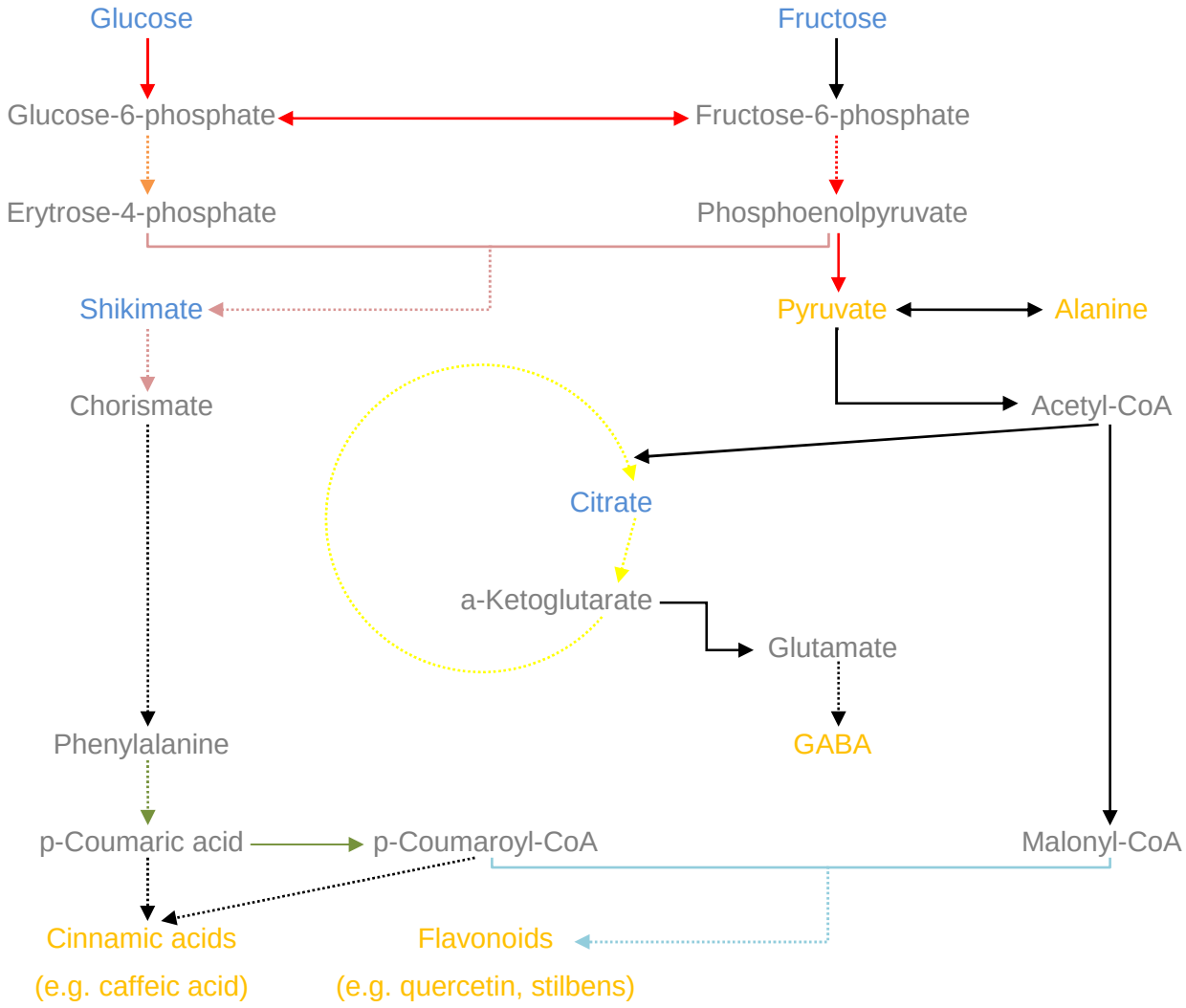

Figure 4.21 – Metabolomic alterations in *Vitis vinifera* cv. Alvarinho diseased leaves. Compounds: decreased (blue); increased (orange), not measured/unassigned (grey): Metabolic pathways: glycolysis (red), pentose phosphate pathway (orange), tricarboxylic acid cycle (yellow), shikimate pathway (pink), phenylpropanoid pathway (green), flavonoids biosynthesis (blue), other pathways (black); dashed arrows omit several steps.

Results showed a huge increase of phenolic secondary metabolites in diseased leaves compared to healthy ones. Plants have developed a number of defence mechanisms in order to cope with imposed stresses, either biotic (as infection by pathogenic organisms) or abiotic (such as temperature, anoxia, heavy metal or UV exposure). One of the main active defence mechanisms is phytoalexin production. Phytoalexins are

compounds with antimicrobial activity that are synthesized *de novo*, after insult recognition (Dixon, 2001). In grapevine, the accumulation of phytoalexins upon fungal infection is one of the best described defence reactions, and the capacity of the plant to produce phytoalexins has been correlated to disease resistance (Bruno and Sparapano, 2006a; Bruno and Sparapano, 2006b; Douillet-Breuil *et al.*, 1999; Figueiredo *et al.*, 2008; Sarig *et al.*, 1997).

Previously, the increase in polyphenols (in particular stilbenes, but also derivatives of cinnamic acids, quercetin and kaempferol) was reported in grape roots, wood, xylem sap, leaves and *calli* of esca-diseased grapevines or infection with *Pch* and other esca-related fungi (Amalfitano *et al.*, 2000; Bruno and Sparapano, 2006a; Bruno and Sparapano, 2006b; Del Rio *et al.*, 2001). In addition, the fungitoxic activity of these phytoalexins was show *in vitro* against several fungi, including the esca-associated fungi *Pch* and *Phaeoacremonium* spp. (Del Rio *et al.*, 2001; Sarig *et al.*, 1997).

The results presented here seem to indicate that the production of these phenolic secondary metabolites in diseased leaves result from the deviation of primary metabolism. The rerouting from primary to secondary metabolism in plants has been documented. When biotic stress is imposed to plants, they respond allocating carbon from primary metabolism to secondary metabolism in order to enhance concentrations or synthesize *de novo* defensive compounds (Gawronska and Golisz, 2006). The decrease in carbohydrates and activation of glycolysis has been reported in plants as part of a defence response to biotic or abiotic stresses; therefore, it has been suggested as a source of energy and carbon skeletons necessary to produce defence-related compounds. An increase in glycolytic intermediates was observed in *Arabidopsis* cells in response to oxidative stress; also, a decrease in glucose and fructose was shown in rice coleoptiles in response to anoxia (Baxter *et al.*, 2007; Kato-Noguchi and Ohashi, 2006). Moreover, glycolysis accompanied by an increase in phenylpropanoid levels were reported in opium poppy, *Arabidopsis* and *Medicago truncatula* cells cultures in response to both biotic and abiotic elicitors (Broeckling *et al.*, 2004; Kim *et al.*, 2007; Zulak *et al.*, 2008). As well, an increase in glycolytic intermediates

and phenylpropanoid compounds were registered in *Arabidopsis* plants in response to cold shock and cold acclimation (Kaplan *et al.*, 2004; Kaplan *et al.*, 2007). Recently, the treatment of *Brassica rapa* leaves with methyl jasmonate led to decreased carbohydrates levels as well as to an increase in phenylpropanoids (including cinnamic acids), both *de novo* production and production of higher levels of existing phenolic compounds (Liang *et al.*, 2006) Glucose mobilization was also detected in tobacco leaves infected with TMV, concomitantly with an increase in phenylpropanoids levels (Choi *et al.*, 2006).

In accordance with the described above, *V. vinifera* cv. Alvarinho diseased leaves show a decrease of carbohydrates together with an increase in phenolic compounds (overview in Figure 4.21).

The results here presented also show an increase of the amount of methanol in diseased leaves (being approximately 29% higher than in healthy leaves). It is known that plants produce and emit a large variety of volatile organic compounds (VOCs). Recently, studies in atmospheric chemistry discovered that methanol was a major component in forest air, and methanol was found to be one of the major VOCs emitted from plant leaves (Fall and Benson, 1996; Galbally and Kirstine, 2002; Nemecek-Marshall *et al.*, 1995). Also, the presence of a free methanol pool was demonstrated in bean leaves and found to be higher in young leaves than in older ones (Nemecek-Marshall *et al.*, 1995).

It has been suggested that methanol may result from several processes occurring in plant cells, such as protein repair mechanisms or lignin degradation by wood-rotting fungi (Fall and Benson, 1996). However, it is thought that the majority of methanol produced by plants, particularly in leaves, results from the activity of pectin methylesterases (PMEs) (Fall and Benson, 1996; Frenkel *et al.*, 1998). PMEs are enzymes that catalyse the demethylation of pectins (specifically polygalacturonans) in plant cell walls (Galbally and Kirstine, 2002; Micheli, 2001; Pelloux *et al.*, 2007). These enzymes are active during processes of growth and development when cell wall structure modifications are needed. It has been suggested that plant

growth might contribute with large amounts of methanol emissions into the atmosphere, as suggested by the generally higher emissions of methanol in young growing leaves than in adult leaves (Galbally and Kirstine, 2002; Hüve *et al.*, 2007; Nemecek-Marshall *et al.*, 1995). Besides plant growth, putative processes in which PMEs may play a role include, among others, seed germination, pollen formation and growth of pollen tube, fruit ripening, cell wall elongation, cell wall adhesion, stem elongation and wood development (Micheli, 2001; Pelloux *et al.*, 2007).

Furthermore, it has been hypothesised that PMEs activity may be involved in defence processes. Methanol emissions suggested to be the result of PMEs activity were shown to be specifically enhanced by herbivore attack on leaves or exposure of wounds to larval oral secretions, leading to the hypothesis that methanol might be more than a waste product of PMEs activity and act itself as a defensive substance, possibly as a signal molecule (Pelloux *et al.*, 2007; Peñuelas *et al.*, 2005; von Dahl *et al.*, 2008). But PMEs activity was suggested to be involved in defence at more levels: after pectin demethylation other enzymes may act on exposed bonds leading to the release of oligogalacturonic acids which may act as elicitors, activating plant defence mechanisms. Also, pectin demethylation originates negatively charged galacturonic acid residues which attract cations, such as Ca^{2+}; Ca^{2+} bridges cross-link adjacent galacturonic acid residues enhancing cell wall resistance (Galbally and Kirstine, 2002; Pelloux *et al.*, 2007). Additionally, PMEs have been suggested to be involved in cell wall strengthening by lignification since the Ca^{2+}-bridged galacturonate residues may bind class III peroxidases which are responsible for lignin polymerization (Pelloux *et al.*, 2007). Because it was already reported that esca-diseased leaves of *V. vinifera* cv. Alvarinho increased their lignin content comparatively to healthy leaves (Felgueiras, 2006), the increased methanol content of diseased leaves here detected might reflect an increase in PMEs activity leading to lignification of cell walls as a defence mechanism against the disease.

Besides the metabolic alterations described above, the diseased leaves also show increased levels of alanine and GABA, when compared to healthy

leaves (Figure 4.21). Although their exact function in defence is not well established, increases in alanine and GABA have been reported in plants in response to several stresses (Allan *et al.*, 2008; Kato-Noguchi and Ohashi, 2006; Mayer *et al.*, 1990; Miyashita *et al.*, 2007; Monlise *et al.*, 2003; Wallace *et al.*, 1984), as well as the accumulation of alanine and/or GABA has been observed in metabolic scenarios as the one described for *V. vinifera* cv. Alvarinho diseased leaves, with decrease of carbohydrates accompanied by increase in phenylpropanoid levels (Broeckling *et al.*, 2004; Choi *et al.*, 2006; Kaplan *et al.*, 2004; Kaplan *et al.*, 2007; Zulak *et al.*, 2008).

GABA is formed from glutamic acid in a reaction catalyzed by glutamate decarboxylase (GAD) in the cytosol. GAD is activated by two mechanisms: cytosolic acidification (GAD's optimum pH is acidic) and increase in cytosolic calcium (through the binding of Ca^{2+}/CaM complexes); both mechanisms often accompany biotic and abiotic stresses, which lead to GABA increase in stressful situations. Then, the enzyme GABA transaminase (GABA-T) can use pyruvate or 2-oxoglutarate to synthesize succinic semialdehyde (SSA), leading to the production of alanine or glutamate, respectively (Fait *et al.*, 2007; Kinnersley and Turano, 2000). Given the pyruvate availability in *V. vinifera* cv. Alvarinho diseased leaves, this might be the mechanism responsible for alanine accumulation. Furthermore, this was already suggested for accumulation of alanine under anaerobic conditions (Mayer *et al.*, 1990).

Several roles have been proposed for these two metabolites. Both alanine and GABA have been proposed as temporary nitrogen reserves (to ensure its availability once cellular conditions change) and GABA has been thought to participate in carbon/nitrogen signalling (Vanlerberghe *et al.*, 1991; Zulak *et al.*, 2008). Also, alanine has been implicated in signalling: it was shown that addition of alanine stimulated gene encoding for stress-protein synthesis in mammalian kidney, to protect cells from injury damage (Monlise *et al.*, 2003). In addition, alanine and GABA are metabolites with compatible solute-like properties. Compatible solutes accumulate during stress and contribute to protein and membrane stabilization as well as to maintain osmotic pressure (Kaplan *et al.*, 2004). Since the decrease in carbohydrate

content might decrease osmotic potential, alanine and GABA increase was suggested to be a response to osmotic potential decrease (Kato-Noguchi and Ohashi, 2006). As the results indicated a decrease of glucose and fructose in *V. vinifera* cv. Alvarinho diseased leaves accompanied by increase in alanine and GABA levels, these two last metabolites might be playing a role in osmotic potential maintenance in diseased leaves. Several other roles have been proposed for GABA: pH regulation (because GAD activity consumes H^+), regulation of plant growth and development, plant defence against herbivory (GABA is an inhibitory neurotransmitter in animals), TCA cycle bypass and signalling molecule (including gene induction) (Kinnersley and Turano, 2000; Shelp *et al.*, 1999). Furthermore, the application of a commercial product containing GABA (AuxiGro^{TM}) to several crops (including grape) led to enhanced resistance to various pathogens; supporting the proposed signalling role of GABA, since it was demonstrated that this product has neither fungistatic nor fungicidal activity (Kinnersley and Turano, 2000).

Besides phytoalexins, plants produce constitutive antifungal substances (including toxins and PR proteins) called phytoanticipins, that can act immediately upon pathogen attack (Harborn, 1999). The compounds quercetin-3-*O*-glucoside and caffeic acid are detected by ^{1}H-NMR in healthy leaves; also, in the previous section (4.2.1), kaempferol-3-glucoside was identified. These compounds might be functioning in healthy leaves as phytoanticipins. The flavonoids quercetin and kaempferol were shown to have antifungal activity (Treutter, 2006). In addition, caffeic acid was implicated in inhibition of germination of *Phytophthora* spp. zoospores, as well as in antibacterial activity against several resistant bacteria (Nascimento *et al.*, 2000; Widmer and Laurent, 2006).

According to the literature, the metabolism of *V. vinifera* cv. Alvarinho diseased leaves reflects an active state of defence against imposed stress – esca disease – with deviation of carbon and energy from carbohydrates to the production of antifungal phenolic compounds.

4.3 - Conclusions

V. vinifera cv. Alvarinho leaves seem to initiate a defence response when in presence of fungi of the esca complex. HPLC analysis of methanolic extracts of healthy, apparently healthy and diseased leaves revealed differences in the phenolic content of these leaves. The application of a PCA to HPLC data indicated differential phenolic production, since it clearly separated healthy and diseased leaves, with apparently healthy leaves clustered in a medial position. The results show that total phenolic production significantly increases in diseased and apparently healthy leaves, comparatively to healthy leaves. Furthermore, the amount of phenolic compounds seems to increase linearly from healthy to diseased leaves, with apparently healthy leaves presenting a medial amount. The compound kaempferol-3-glucoside was identified and its amount quantified: again, results showed a significant increase in diseased and apparently healthy leaves, comparatively to healthy leaves, with apparently healthy leaves showing a medial amount of kaempferol-3-glucoside. Thus, the results suggest that *V. vinifera* cv. Alvarinho leaves are capable of producing defensive phenolic compounds in the presence of esca and that the increased production of phenolic compounds is activated before the appearance of foliar symptoms.

But the defence response includes several metabolic alterations: although production of phenolic compounds is important, it is not the only metabolic alteration in the course of esca disease response. Besides the huge increase of phenolic compounds in diseased leaves, compared to healthy leaves, a decrease of carbohydrates was also detected, suggesting that diseased leaves are rerouting carbon and energy from primary to secondary

metabolism. Other metabolic alterations detected were increased levels of methanol, alanine and GABA in diseased leaves, which might be the result of the activation of other defence mechanisms.

CHAPTER 5 - CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the previous page: illumination by Jean Bourdichon, from *Horae ad usum romanum – Grandes heurs d'Anne de Bretagne* (1503-1508), Tours – France; Bibliothèque nationale de France, Département des Manuscrits, Division occidentale (cote Latin 9474, folio 156). Accessed from Mandragore, base des manuscrits enluminés de la Bibliothèque nationale de France (http://mandragore.bnf.fr/html/accueil.html) on 12th January 2009.

Esca is a destructive disease of complex aetiology affecting grapevines worldwide. Although it is not a new disease, it is now considered a major problem in viticulture since its incidence had a huge increase worldwide in the last two decades. Some regions are already severely affected while the disease incidence continues to increase in several countries, including Portugal. Until now, no effective method could be developed to control the esca. This scenario has been leading to important losses in longevity and productivity of grapevines and in wine quality, as well as carrying great economic costs for replanting vineyards. In RDVV, esca and young grapevine decline has also been leading to important losses in wine production. The disease is widespread all over the region, is affecting all varieties of *Vinho Verde* grapevines, and affects both old and young plants.

In order to contribute to a better understanding of the disease, an *in vitro* model was established, using leaf explants of *V. vinifera* cv. Vinhão (a variety considered less susceptible to esca and therefore suggesting that this variety has defence mechanisms that enable coping with the disease) to create *calli* and cells suspension cultures.

Pch extract elicitation of *V. vinifera* cv. Vinhão cells cultures led to an increased phenolic production, including the *de novo* production of the viniferin type compounds ε-viniferin-2-glucoside, ε-viniferin-glucoside and a polymer of two ε-viniferin molecules. Viniferins were already described as antifungal compounds. Also, the suspension cultures allowed the study of the effect of the signalling molecules SA and MeJ on defence. Particularly, MeJ showed to have potential in grapevine protection since it led to an increase in stilbenic and anthocyanin production; plus MeJ induced the *de novo* production of the same viniferin type compounds as *Pch* elicitation.

Besides, *Pch* extract elicitation of *V. vinifera* cv. Vinhão cells cultures resulted in a biphasic oxidative burst, typical of incompatible interactions. The influence of priming with the phytohormones SA and MeJ in the oxidative burst was also analysed. Although ROS accumulation seems to be potentiated in the first phase of the burst by SA priming, priming with both SA and MeJ seems to be obstructive to ROS accumulation during the second phase of the burst. Cells suspensions also allowed to study NADPH oxidase involvement in the oxidative burst, leading to the conclusion that this enzyme is the primary ROS font in this process. The putative role of catalase and peroxidase enzymes in the oxidative burst was also analysed. The results indicated that catalase activity is not altered by *Pch* elicitation and, therefore, other types of detoxifying mechanisms must be regulating ROS burst. Regarding peroxidase, results indicated that this enzyme may play a role during the second phase of the oxidative burst. The influence of Ca^{2+} in the oxidative burst was also evaluated, and it was concluded that Ca^{2+} is necessary to oxidative burst occurrence and that the Ca^{2+} channel types from which Ca^{2+} influx is originated are also important to induce oxidative burst. Furthermore, Ca^{2+} availability also seems to be necessary for the activation of NADPH oxidase.

In addition, *Pch* extract elicitation of *V. vinifera* cv. Vinhão cells cultures was capable of inducing the expression of genes encoding pathogen-related proteins (PR-6, PR-10, GLUC and CH3) and genes encoding enzymes involved in the octadecanoid (LOX) and phenylpropanoid (PAL and STSY) pathways.

Thus, *Pch* extract elicitation of cells cultures could induce several defence mechanisms (phytoalexin production, oxidative burst, Ca^{2+} signalling, NADPH oxidase activity and defence-related gene expression) indicating that *V. vinifera* cv. Vinhão cells suspensions could be an important tool to study esca disease, since they allow to selectively study host defence response to esca-related fungi without interference of external factors in a short period of time (2-3 weeks), leading to reliable results. Also, occupying a small space, cells suspension cultures make possible the analysis of a large number of culture flasks and/or cultivars.

In conclusion, *V. vinifera* cv. Vinhão cells suspension cultures offer a simple, rapid and selective way to investigate the interaction between *Vitis* and esca-related fungi, therefore representing a valuable model to study esca disease. Further understanding of which defence mechanisms are triggered in esca resistant varieties might lead to strategies to fight the disease in field plants or, at least, to minimize the negative effects of esca. Also, cells suspension cultures could allow the study of possible effects of pesticides on grapevine cells, or allow to study how application of abiotic elicitors or biological control agents activate defence responses against further pathogen attack, in a rapid and controlled manner. The future development of a plantlet *in vitro* model might allow the study of the physiological changes induced by the disease, as well as allow the investigation of defence mechanisms (particularly activation of defence-related genes transcription and production of defensive compounds) triggered in a more complex system, with different tissue types. It may also allow screening different treatments in controlling or minimizing the negative effects of the disease, in a controlled manner and more rapidly than in field studies.

Metabolomic studies of field material were also performed. Leaves of *V. vinifera* cv. Alvarinho (a variety considered susceptible to esca) were analysed in order to observe if, in spite of its susceptibility, the production of defensive compounds were activated *in planta*.

The results indicate that *Vitis vinifera* cv. Alvarinho leaves seem to initiate a defence response when in presence of fungi of the esca complex. HPLC analysis of methanolic extracts of healthy, apparently healthy and diseased leaves revealed differences in the phenolic content of these leaves. The application of a PCA to HPLC data indicated differential phenolic production, since it clearly separated healthy and diseased leaves, with apparently healthy leaves clustered in a medial position. The results show that total phenolic production significantly increases in diseased and apparently healthy leaves, comparatively to healthy leaves. Furthermore, the amount of phenolic compounds seems to increase linearly from healthy to diseased leaves, with apparently healthy leaves presenting an intermediate amount. The compound kaempferol-3-glucoside was identified and its amount

quantified: again, results showed a significant increase in diseased and apparently healthy leaves, comparatively to healthy leaves, with apparently healthy leaves showing an intermediate amount of kaempferol-3-glucoside. Thus, the results suggest that *V. vinifera* cv. Alvarinho leaves are capable of producing defensive phenolic compounds in the presence of esca and that the increased production of phenolic compounds is activated before the appearance of foliar symptoms.

Also, the results presented here demonstrate the great potential of NMR spectroscopy-based metabolomic studies applied to the study of esca disease. Although production of phenolic compounds is important, it is not the only metabolic alteration in the course of the defensive response towards esca. Besides the huge increase of phenolic compounds in diseased leaves, compared to healthy leaves, a decrease of carbohydrates was also detected, suggesting that diseased leaves are rerouting carbon and energy from primary to secondary metabolism. Other metabolic alterations detected were increased levels of methanol, alanine and GABA in diseased leaves, which might be the result of the activation of other defence mechanisms.

Further identification of the metabolites involved in the defence response, possibly using LC-NMR, could contribute to a better understanding of defence responses triggered. Future metabolomic analysis of healthy and esca infected leaves of resistant and susceptible grapevine varieties would allow the comparison between metabolic alterations provoked by esca in resistant and susceptible varieties, contributing to a better understanding of the disease, and perhaps indicating the direction to the development of a treatment. Transcriptomic analyses could also give a positive contribution to this study, leading to a greater depth in the understanding of esca and how and/or how fast resistant and susceptible varieties react to the disease. Meanwhile, the application of PCA analysis to metabolomic data (resulting from HPLC or NMR) could become a useful method for quality control of vineyards or for analysing the presence or course of esca disease.

Undoubtedly, the metabolic responses described previously are esca-related given the typical esca symptoms and the isolation of fungi of the esca

complex in the boughs presenting diseased leaves; however, they may not be esca-specific responses since in field material was exposed to several other biotic and abiotic factors. Given the intrinsic complexity of the disease a profile to a proper control to use in these studies remains undefined. As such, to further obtain more reliable results, studies investigating esca-specific responses should be conducted for several years in the same vines; also, the presence of other pathogenic fungi should be screened and better control plants should be encountered (not relying only in the absence of external foliar symptoms). In addition, studies in greenhouse plants would be advantageous since they allow a better control of external abiotic conditions.

Because esca results from a still unexplained interaction between esca-related fungi, grapevines and environmental factors and, in spite of all efforts no effective treatment was developed until now, it seems now established that only a better understanding of the disease may lead to the development of effective control methods against esca.

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